**Biochemical Analysis Of Microsomal NADPH-Cytochrome P450 Oxidoreductase and Carboxylesterase.**

This thesis is my own composition and describes a project carried out by myself; experiments performed by other people are appropriately acknowledged.

Graeme Cameron Murray Smith
PhD.
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1994.

To Stephanie

Hypotheses are nets. He who casts catches.

Novalus.

**Acknowledgements**

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## Abbreviations

| | |
|---|---|
| A | adenosine |
| 3-AcPyADP | 3-acetylpyridine adenine dinucleotide phosphate |
| APS | ammonium persulphate |
| ATP | adenosine 5' triphosphate |
| A. U. | absorbance unit |
| BNPP | bis-nitrophenylphosphate |
| b.p. | base pair |
| BSA | bovine serum albumin |
| C | cytosine |
| $^{o}C$ | degrees celcius |
| C-terminus | carboxyl-terminus |
| cDNA | complementary DNA |
| CIP | calf intestinal phosphatase |
| CO | carbon monoxide |
| con A | concanavalin A |
| cyt c | cytochrome c |
| Da | Dalton |
| DCIP | 2, 6-dichloroindophenol |
| dATP | 2' deoxyadenosine 5' triphosphate |
| dCTP | 2' deoxycytosine 5' triphosphate |
| dGTP | 2' deoxyguanosine 5' triphosphate |
| dNTP | 2' deoxynucleotide 5' triphosphate |
| dTTP | 2' deoxythymidine 5' triphosphate |
| ddNTP | 2' 3' dideoxynucleotide 5' triphosphate. |
| $dH_20$ | distilled water |
| $ddH_20$ | double distilled water |
| DMF | N, N-dimethylformamide |
| DMSO | dimethyl sulphoxide |
| DNA | deoxyribonucleic acid |
| ds | double stranded |
| DTT | dithiothreitol |
| EDTA | ethylenediamine tetraacetic acid |
| ELISA | enzyme linked immunosorbent assay. |
| ER | endoplasmic reticulum |
| EtBr | ethidium bromide |
| FAD | flavin adenine dinucleotide |

| | |
|---|---|
| FMN | flavin mononucleotide |
| FPLC | fast performance liquid chromatography |
| FNR | ferredoxin $NADP^+$ reductase |
| G | guanosine |
| g | gram |
| GST | glutathione S-transferase |
| HRP | horseradish peroxidase |
| IC | inhibitory constant |
| IgG | immunoglobulin G |
| IPTG | isopropyl-β-D-thiogalactopyranoside. |
| kb | kilobase |
| kDa. | kiloDalton |
| mRNA | messenger RNA |
| Mw | molecular weight |
| $NADP^+$ | β-nicotinamide adenine dinucleotide phosphate (oxidised form) |
| NADPH | β-nicotinamide adenine dinucleotide phosphate (reduced form) |
| NaPPi | sodium pyrophosphate |
| NOS | nitric oxide synthase |
| OD | optical density |
| OLB | oligo labelling buffer |
| ompA | outer membrane protein A |
| OPD | o-phenylenediamine dihydrochloride |
| ORF | open reading frame |
| $^{32}P$ | a β-emitting radioactive isotope of phosphorous |
| P450 | cytochrome P450 |
| PAGE | polyacrylamide gel electrophoresis |
| PBC | primary biliary cirrhosis |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| pH | negative log of hydrogen ion concentration |
| PMSF | phenyl-methyl-sulphonyl fluoride |
| PVDF | polyvinyl difluoride |
| RBS | ribosomal binding site |
| red | reductase |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| rpm | revolutions per minute |

| | |
|---|---|
| $^{35}S$ | a β-emitting radioactive isotope of sulphur |
| SD | Shine Dalgarno |
| SDS | sodium dodecyl sulphate |
| SOD | superoxide dismuthase |
| ss | single stranded |
| SSC | 150 mM sodium chloride, 15 mM sodium citrate |
| T | thymidine |
| TEMED | N, N, N', N'- tetramethyl-ethylenediamine |
| TFA | Trifluoroacetyl |
| Tris | 2-amino-2-hydroxymethyl-propane-1,3-diol |
| Tween 20 | polyoxyethylene (20)-sorbitan-monolaurate |
| U | uracil |
| UV | ultraviolet |
| V | volt |
| v/v | volume per unit volume |
| W | watts |
| w/v | weight per unit vloume |
| XGAL | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |
| *Pfu* | *Pyrococcus furiosus* |

## Table of amino acids.

| Name | Abbreviations | |
|---|---|---|
| Alanine | Ala | A |
| Arganine | Asn | R |
| Asparagine | Asp | D |
| Aspartic Acid | Asn | N |
| Cysteine | Cys | C |
| Glutamic Acid | Glu | E |
| Glutamine | Gln | Q |
| Glycine | Gly | G |
| Histidine | His | H |
| Isoleucine | Iso | I |
| Leucine | Leu | L |
| Lysine | Lys | K |
| Methionine | Met | M |
| Phenylalanine | Phe | F |
| Proline | Pro | P |
| Serine | Ser | S |
| Threonine | Thr | T |
| Tryptophan | Trp | W |
| Tyrosine | Tyr | Y |
| Valine | Val | V |

# UNIVERSITY OF EDINBURGH

## ABSTRACT OF THESIS (Regulation 3.5.10)

*Name of Candidate* Graeme Cameron Murray Smith

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The endoplasmic reticulum is a rich source of enzymes involved in the metabolism of xenobiotics/drugs. Two enzymes, NADPH-cytochrome P450 oxidoreductase and a carboxylesterase, have been studied.

The microsomal flavoprotein NADPH-cytochrome P450 oxidoreductase functions as a 'shuttle' in the transfer of electrons from NADPH to the physiological acceptors cytochromes P450. Exogenous acceptors, such as paraquat and quinone containing compounds, can also be reduced. To assess the role of P450 reductase in free radical cytotoxicity via redox cycling of compounds, the rat enzyme was expressed in both *S. typhimurium* and *E. coli*. *S.typhimurium* expressing P450 reductase were found to be more sensitive to the cytotoxic effects of paraquat and menadione but not to the anticancer drug doxorubicin. Similarly, the growth inhibitory effects of paraquat and menadione were found to be enhanced in the P450 reductase expressing bacteria. Using superoxide dismutase and catalase as markers of the oxidative stress inducible regulons *soxRS* and *oxyR*, it was found that in *E. coli* there was no difference in the induction of these enzymes between the expressing and non-expressing strains.

P450 reductase contains the cofactors FAD and FMN as well as being able to bind NADPH and interact with P450s and membranes. In order to probe the evolution and function of this protein, proposed domains of the enzyme have been expressed in *E.coli* with a 6x-Histidine tag and purified by a single step using nickel-agarose chromatography. The putative FMN and FAD/NADPH domains were found to bind their respective cofactors independently. However, FMN and FAD binding in these domains was found to be less than the expected ratio of 1 mole flavin: 1 mole protein. The FAD/NADPH domain catalysed the one electron reduction of a variety of compounds. This portion of the protein was unable to efficiently reduce cytochrome *c* or P450. However, the FMN and FAD/NADPH domains could be combined to produce a functional system that was active in the reduction of cytochrome c and cytochrome P450 (as judged by the oxidation of the CYP1A1 substrate 7-ethoxyresorufin). The FMN domain containing the hydrophobic membrane anchor was a potent inhibitor of reconstituted monooxygenase activity.

A human liver carboxylesterase was purified to electrophoretic homogenity after a two step procedure involving DEAE-cellulose and hydroxylapatite chromatography. The purified protein had an apparent molecular weight of 59kDa. and was glycosylated with mannose residues. The enzyme had high specific activity to a variety of carboxylesterase substrates and was inhibited by the serine hydrolase inhibitor, phenylmethylsulphonyl fluoride, and the specific carboxylesterase inhibitor *bis*-4-nitrophenylphosphate. The amino-terminal sequence showed high homology to a carboxylesterase cloned from human alveolar macrophages and human liver, as well as carboxylesterases purified from other mammalian species. Western blot analysis with an antibody to the pure protein showed that the enzyme was only expressed in the liver and its level varied by only 2.5 fold across a panel of human liver microsomes. The carboxylesterase was shown to be a target for autoantibodies in the sera of patients with the immune mediated disease halothane hepatitis. Immunohistochemical analysis found the protein to be located in the centrilobular region of liver sections.

## Publications arising from this research.

Smith, G. C. M., Kenna, J. G., Harrison, D. J., Tew, D. and Wolf, C. R. (1993) Autoantibodies to a human hepatic microsomal carboxylesterase in patients with halothane hepatitis. The Lancet. **342**, 963-964.

Smith, G. C. M., Tew, D. and Wolf, C. R. (1994) Dissection of NADPH cytochrome P450 oxidoreductase into distinct functional domains. Proc. Natl. Acad. Sci. **91**, 8710-8714.

Smith, G. C. M., Tew, D. and Wolf, C. R. Purification and characterisation of a human microsomal carboxylesterase. Submitted to the Biochemical Journal.

## Published Abstracts

Smith, G. C. M., Tew, D. and Wolf, C. R. (1992) Purification and characterisation of a human microsomal carboxylesterase implicated in halothane induced hepatitis. J. Basic and Clin. Physiol. and Pharmacol. **3**, 281.

Smith, G. C. M., Simula, A. P., Van der Drift, P., Kasper, C. B., Tew, D. and Wolf, C. R. (1992) Cytochrome P450 oxidoreductase expressed in *E.coli* and *S.typhimurium* for studying one electron acivation reactions. J. Basic and Clin. Physiol. and Pharmacol. **3**, 282.

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# Chapter 1: Introduction.

## 1.1 General Aspects of Drug metabolism.

### 1.1.1 Historical Perspectives.

The origins of drug metabolism are to be found in the first half of the nineteenth century. Later in that century all the major pathways of drug metabolism were elucidated i.e. oxidation; reduction; hydrolysis; conjugation with glycine, sulfuric acid and glucuronic acid; acetylation and mercapturic acid synthesis (reviewed by Bachman and Bicknell, 1985). The discovery by Ure (1841) on the biotransformation of benzoic acid into hippuric acid may be considered as the birth of drug metabolism.

Conjugation reactions were the first to be discovered since the conjugating agent endows the xenobiotic with distinct physicochemical properties which facilitate their separation. On the other hand oxidative or phase I metabolites belong to a great variety of chemical classes, have little in common and their identification was difficult with the methodologies available at that time. The early studies employed were based on administering the xenobiotic to man or an animal and following its fate through analysis of urine. The anti-malarial quinine and the analgesic morphine which have been used effectively for centuries served as model compounds for many of the pioneering studies.

In early drug metabolism studies the chemical compound rather than the organism was at the centre of the scientific interest. The fact that a foreign compound was altered because it had been introduced into a system which was able to perform chemical reactions was looked upon more as a biochemical curiosity rather than a physiologically meaningful process (Bachman and Bicknell, 1985). However, in the late nineteenth century this attitude began to change when it was found that metabolites of aromatic compounds were much less toxic than their parents (Baumann, 1876; Nencki and Boutmy, 1892). This led to the synonym 'detoxication mechanisms' which preceded the modern term of 'drug metabolism'. The former term was changed after the discovery of toxication reactions in the 1930's (Kinosita, 1937 and Sasaki and Yoshida, 1935). Together with his earlier work and a later publication (Williams, 1947; Williams, 1959), Williams brought together the main theories that had been accumulating for over fifty years

about the physiolgical role of detoxication mechanisms/ drug metabolism. These theories included; 'The Chemical Defense Hypothesis' of Sherwin (1922) which described oxidations, reduction and conjugations as rendering drugs more soluble thus aiding their excretion and 'The Decreased Lipophilicity Hypothesis' of Schuller (1925) which stated that conjugations lead to the decreased lipophilicity of the drug which in turn results in altered distrubution, shifting the drug to the extracellular phase. These early theories resulted in Williams's model of the "Biphasic Metabolism of Drugs', which is outlined below in section 1.1.2.(Williams 1959).

The methodology available at a given time dictates what can be achieved in the study of a scientific discipline. As the methodology improves we are able to study the subject of interest in increasing detail, thus following a basic reductionist philosophy. This is clearly exemplified in the study of drug metabolism. Initial studies looked at the fate of a xenobiotic by the whole organism and then eventually specific organs. It wasn't until the 1950s with the development of isolation techniques of subcellular compartments that the field of drug metabolism really took off (see section 1.1.4). The advancement of protein purification techniques further aided the study of xenobiotic metabolism in that the specific enzymes, that were catalysing the reactions, could be isolated and studied. Now in the age of recombinant DNA technology we are able to clone the cDNAs and genes for the enzymes that are involved in drug metabolism. Through the use of heterologous expression systems functional and structural analysis of individual enzymes can now be studied in more detail. Furthermore, by studying gene promoter sequences the regulation and tissue specific expression of these enzymes can be examined.

### 1.1.2 The Biphasic Metabolism Of Drugs.

The present day views of drug metabolism are highlighted in Figure 1.1 and, as described above, first outlined by Williams (1947, 1959). The first phase can result in the inactivation of a drug, the conversion of an initially inactive compound into an active drug or the conversion of an active drug into another active drug. The second phase of drug metabolism consists of conjugating reactions, most of which convert active compounds into inactive excretory products. Phase I reactions generally bring about the introduction of a suitable functional group into the drug molecule. This changes the drug, in

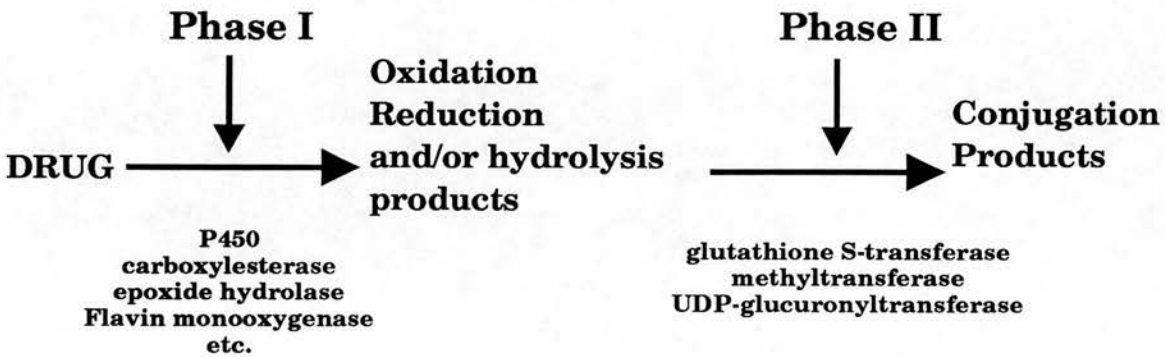



**Figure 1.1. The biphasic metabolism of drugs.**

most cases, to a more polar form and hence a more readily excretable form. The product of phase I metabolism may then act as the substrate for phase II metabolism, resulting in conjugation with endogenous substrates, increased water solubility and polarity and drug elimination from the body in either the urine or bile.

Oxidation, reduction, hydrolysis, hydration and isomerisation are all examples of phase I metabolism. These reactions are mediated by a number of enzyme systems including alcohol dehydrogenase, xanthine oxidase, the microsomal flavin containing monooxygenase, carboxylesterase, epoxide hydrolase and the cytochrome P450 monooxygenase system. This latter system plays a central role in the oxidative metabolism of structurally diverse xenobiotics and also plays an important role in the synthesis and degradation of many endogenous biosynthetic intermediates. This system will be discussed in more detail in section 1.2.1. Studies on NADPH cytochrome P450 oxidoreductase, an integral component of the P450 monooxygenase system will be the basis of chapters 3 and 4. Serum as well as microsomal carboxylesterases are involved in the hydrolysis of ester containing compounds. These enzymes are not only important for the inactivation of drugs but in the metabolism of pro-drugs into their active moieties. The purification and characterisation of a human hepatic microsomal carboxylesterase is the basis of the work presented in chapter 5.

Phase II metabolism or conjugation also involves a diverse group of enzymes acting on a diverse group of compounds. Both foreign compounds and many natural metabolites of the body containing suitable chemical groups such as OH, $NH_2$ and COOH, the products of phase I metabolism, will undergo synthetic conjugation reactions. The conjugated products of phase II metabolism are more water soluble and excreted in the the bile or urine. Examples of phase II reactions include glucuronidation, catalysed by the UDP-glucuronyl trnsferases; glycosidation catalysed by the UDP-glycosyltransferases; sulfation, catalysed by the sulfotransferases; methylation, catalysed by the methyltransferases; acetylation, catalysed by the acetyltransferases and glutathione conjugation, catalysed by the glutathione S-transferases.

The further metabolism of glutathione conjugates which leads to cysteine conjugates and mercapturic acids is considered by some workers as an additional phase of metabolism. For example, metabolism of glutathione conjugates may take place by the intestinal microflora which can result in

reabsorption of the compound and further metabolism in the liver. More recently the term "phase III" has been applied to describe the elimination of glutathione conjugates from the cell by an ATP-dependent glutathione S-conjugate export pump (Ishikawa, 1993). Whether these examples can be considered as a further phase of drug metabolism is open to debate.

### 1.1.3. Localisation of Drug Metabolic Pathways.

In early drug metabolism studies, foreign compounds were administered to animals or human subjects and reaction products looked for or identified in the urine. The localisation of drug metabolic pathways initially involved techniques such as hepatectomy of laboratory animals and perfusion of the liver and kidneys (reviewed by Conti and Bicknell, 1977). These original studies led to the conclusion that the liver was the major site of drug metabolism. In a quantitative sense the liver is the main organ responsible for both phase I and phase II drug metabolism reactions, although this is by no means the only organ involved. Drug localisation and metabolism in a given tissue is dependent on many factors including the physico-chemical properties of the drug, chemical composition of the organ and the presence of uptake mechanisms which may allow the drug to be 'trapped'. Other organs where metabolic reactions have been observed include skin, gastrointestinal tract, gastrointestinal flora, lung, blood, brain, kidney and placenta.

Most of our fundamental knowledge regarding the molecular mechanisms of drug metabolism have been derived from studies on the liver. The development of systems for the disruption of mammalian cells (Potter and Elvehjem, 1936) and of centrifugal methods for the preparation of enzymatically functional cellular organelles (Claude, 1941; Scheider, 1949) facilitated approaches to examining *in vitro* metabolism of xenobiotics. The first study utilising subcellular organelles was carried out by Mueller and Miller (1948). They showed that N-demethylation and aromatic ring hydroxylation occurred on aerobic incubation of dimethyl-1-4-aminoazobenzene with pyridine nucleotide treated rat homogenates.

The majority of phase I reactions are carried out by enzymes located in the endoplasmic reticulum of hepatic cells (Brodie*et al.*, 1955; Gillette, 1963). On homogenisation of cells the endoplasmic reticulum is disrupted giving rise to small vesicles. These can be separated from the homogenate by high speed centrifugation and give rise to the fraction known as microsomes. Phase II

reactions are found to occur predominantly in the cytoplasm. The one major exception to this being glucuronidation which is carried out by the UDP-glucuronosyltransferases in the endoplasmic reticulum.

### 1.1.4 The endoplasmic reticulum.

Electron microscopy studies of hepatocytes reveal the endoplasmic reticulum to be present throughout the cytoplasm as an extensive network of tubules, vesicles and lamellae (Loud, 1968; Weibel *et al.*, 1969). In the liver it is found that the morophological characteristics of the endoplasmic reticulum vary from region to region (Loud, 1968). On average the endoplasmic reticulum occupies 15% of the total cell volume and has a volume 2.5 times that of the nucleus and 65% that of the mitochondria. Compositional studies of rat liver microsomes reveal the membrane of the endoplasmic reticulum to be approximately 70% protein and 30% lipid (of which 85% is phospholipid) by weight (Glaumann and Dallner, 1968). Microsomes have also been found to contain cholesterol (0.6 mg/g liver), triglycerides (0.5 mg/g liver), small amounts of cholesterol esters, free fatty acids and vitamin K (Depierre and Dallner, 1975). Located on the endoplasmic reticulum are ribosomes and a large number of enzyme and enzyme systems. Among the functions of the endoplasmic reticulum other than drug metabolism (see sections above and below) are the synthesis and transport of a number of proteins, including glycoproteins and lipoproteins, synthesis of cholesterol, steroid hormones, prostaglandins, phospholipids and triglycerides as well as glycogen breakdown (Depierre and Dallner, 1975). The endoplasmic reticulum is well known to respond to phenobarbital, carcinogens and other xenobiotics by increasing its volume and surface area (about 2 fold in the case of phenobarbital), (Straubli *et al.*, 1969). Certain regions of the endoplasmic reticulum bear ribosomes bound to the cytoplasmic surface (rough endoplasmic reticulum) and are concerned with the synthesis of proteins secreted from the cell and of proteins destined for incorporation into the endoplasmic reticulum. Other regions are devoid of ribosomes (smooth endoplasmic reticulum) and although not capable of synthesising proteins are involved in the other numerous aspects of this membrane system.
Unlike other organelles, such as mitochondria and lysozymes, the endoplasmic reticulum is extensively disrupted upon gentle homogenisation of the liver. This breakage, into the closed vesicles termed microsomes, does

not seem to be a purely mechanical process, but to involve an active 'pinching off' process (Depierre and Dallner, 1975). Microsomes have ribosomes on their outer surface and contain secretory proteins such as albumin (known to be present in the lumen of the endoplasmic reticulum) *in situ*. Thus the outside of the microsome corresponds to the cytoplasmic surface of the endoplasmic reticulum, while the luminal surface is inside. To date it has not yet been possible to obtain microsomes with the opposite orientation.
The endoplasmic reticulum has been a rich source in the study of protein biosynthesis; lipid metabolism; prostaglandin, cholesterol and steroid biosynthesis; and for the study of drug metabolism. The following sections will now deal with the major drug metabolising systems found within the endoplasmic reticulum. Particular emphaisis will be put on the cytochrome P450 monooxygenase system.

## 1.2 Drug metabolising systems of the endoplasmic reticulum.

### 1.2.1. Cytochrome P450.

By far the most studied group of drug metabolising enzymes located on the endoplasmic reticulum is the cytochrome P450 multisubstrate mixed function monoxygenase system. The key enzyme components of the microsomal system are the flavoprotein NADPH cytochrome P450 oxidoreductase (P450red), the cytochrome P450s and cytochrome $b_5$. P450s are also present in the mitochondria. However, these are quite distinct from their microsomal counterparts in that they function with a two component system comprising adrenodoxin and adrenodoxin reductase. The major P450s of the mitochondria are the cholesterol side chain cleavage enzyme and steroid 17 $\beta$ hydroxylase (reviewed by Fevold, 1983). These enzymes are associated with severe genetic defects in cortisol biosynthesis (Miller and Levin, 1987). The microsomal P450s will be dealt with in this section while section 1.2.2 will discuss P450red with section 1.2.3 dealing with how these enzymes function together to produce the electron transfer system responsible for the oxidation of xenobiotics.
A reduced pigment of hepatic microsomes that had an absorption band with a $\lambda$max at 450nm after binding carbon monoxide was first identified by Klingenberg (1958) and Garfinkel (1958). This pigment was further characterised as a hemoprotein of the b-type by Omura and Sato (1962).

Through photochemical action spectra, Cooper *et al.*, (1965) showed that this hemoprotein was involved in the oxidation of drugs and steroids. The solubilisation and resolution of the components of this system from microsomal membranes and the reconstitution of an active complex containing P450, P450red and phosphatidylcholine permitted the purification and characterisation of these constitutents (Lu and Coon, 1968; Strobel *et al.*, 1970).

Animals, plants and microorganisms have been found to contain P450s and in mammals the enzyme system has been found in all tissues examined so far (Porter and Coon, 1991). P450s have been stated to be the most versatile catalyst known (Porter and Coon, 1991). Among the compounds metabolised by P450s are (a) endogenous compounds such as fatty acids, prostaglandins, steroids and ketones; (b) carcinogens, including polycyclic aromatic hydrocarbons, nitrosamines, hydrazines and arylamines and (c) a plethora of clinically important drugs such as the tricyclic antidepressants, antibiotics, cardiovascular agents, anti-inflammatory compounds and immunosuppressants (Gonzalez, 1989). Reactions that have been shown to be mediated by P450s include aromatic and aliphatic hydroxylation, epoxidation, peroxidation, deamination, desulphuration, dehalogenation and reduction (Porter and Coon, 1991; Guengerich,1991). The basic and unifying reaction carried out by P450s is shown below, with RH representing the substrate;

$$RH + O_2 + NADPH + H^+ \longrightarrow ROH + H_2O + NADP^+ \quad \text{(Equation 1)}$$

To date 221 P450 cDNAs have been isolated which were obtained from 31 eukaryotes, including 11 mammalian species, and 11 prokaryotes (Nelson *et al.*, 1993). It has been estimated that greater than thirty P450s will be expressed at any one time in a mammalian species and many of these are concurrently expressed in a single tissue (Guengerich, 1991).

To aid in the distinction of particular P450 isoforms and to allow isoforms from different species to be logically related, a nomenclature system for this supergene family has been devised (Nelson *et al.*, 1993). The basis for this system is alignments of the amino acid sequences deduced from P450 cDNAs (Nebert *et al.*, 1991; Nelson *et al.*, 1993). P450 enzymes are denoted by the root 'CYP' and distinguished from other families by an arabic numeral. There is then a letter describing the subfamily and finally another arabic numeral denoting the isoform of that subfamily. The mammalian P450 families 1 to 3,

with the possible inclusion of family 4 are involved in the metabolism of xenobiotics The individual enzymes of these families are capable of metabolising a wide range of substrates (Guengerich, 1991). A further six families are involved in the metabolism of of steroids and bile acids. These, in comparison to the xenobiotic metabolising forms, have very specific substrates and reactions.

The diversity of the P450 system has been proposed to have occurred as an adaptive response to environmental challenge. The drug metabolising P450s are thought to have appeared at more advanced stages of evolution in order to protect against toxins in the environment (Nebert, 1979, 1992). This adaptive response is analagous to the response of the immune system when challenged by a foreign compound. The number of man-made 'environmental chemicals' has been estimated at greater than 200,000 (Porter and Coon, 1991). Most of these are thought to be potential substrates for P450 or to act as inhibitors or inducers of the various isoforms.

An area of research that has recieved much attention is the regulation of P450 expression (reviewed by Gonzalez, 1989; Okey, 1990). Considerable diversity in the mechanisms of regulation of these enzymes exists. The most common means of regulation is transcriptional, but post-transcriptional mechanisms include mRNA stabilisation and post-translational modifications. Stabilisation or degradation of P450 protein may be mediated through changes in the phosphorylation state of the enzyme.

Structural studies on mammalian P450s have, until recently, been based on the three dimensional structure for the soluble prokaryote P450 (CYP101, P450cam) from *Pseudomonas putida* (Poulos *et al.*, 1987). More recently the structure of the P450 portion of the soluble P450/P450 reductase fusion protein from *Bacillus megaterium* has been determined (Ravichandrin *et al.*, 1993). This has been proposed to be a better model for the structure of mammalian P450s since it shares homology with the P450s of family 4. Mammalian P450s are believed to have a membrane attachment region at the $NH_2$-terminus of the protein, while the rest of the protein exists on the cytosolic side of the endoplasmic reticulum (Nelson and Strobel, 1988).

### 1.2.2 NADPH cytochrome P450 oxidoreductase

The first reports concerning what is now known as NADPH cytochrome P450 oxidoreductase (P450red) described the purification of a flavoprotein that was involved in the reduction of cytochrome c (Haas, 1940; Horecker, 1950). This early work showed that NADPH was the source of reducing equivalents. The yeast enzyme was found to contain FMN as a prosthetic group (Haas, 1940) while the pig liver enzyme had FAD as its cofactor (Horecker, 1950). The flavin groups were shown to be essential for the reduction of cytochrome c. Phillips and Langdon (1962) and Williams and Kamin (1962) subsequently found the enzyme to be located in the endoplasmic reticulum. However, the physiological substrate for P450red was unknown (since cytochrome c is found in the mitochondria). Furthermore, the involvement of the flavins in the electron transport process was not understood and the exact flavin content of P450red was unknown. More than ten years later the protein was finally identified as containing one molecule each of FMN and FAD (Iyanagi and Mason, 1973; Yasukochi and Masters, 1976). Until the recent discovery of the nitrc oxide synthases (Bredt *et al.*, 1991), P450red was a unique mammalian enzyme in that it contained both of these two flavins as prosthetic groups.

Indirect support for the role of P450red in microsomal hydroxylation and demethylation reactions came about when it was found that cytochrome c reductase activity could be concomitantly induced (Ernster and Orrenius, 1965). Direct evidence for the involvement of P450red in microsomal hydroxylation reactions came from reconstitution of laurate ω-hydroxylase activity from detergent solubilised preparations of P450, P450red and phosphatidylcholine (Lu and Coon, 1968). The early difficulties in identifying a physiological role for the reductase was due to the methods of purification employed, as these studies used trypsin or lipase treatment (Horecker, 1950 and Williams and Kamin, 1962). This resulted in a 68 kDa. protein which had lost approximately 5 kD. from the $NH_2$-terminal region. This region is responsible for interactions with both P450 and phospholipid (Black and Coon, 1982). The proteolytically cleaved enzyme is unable to reconstitute with P450s but is still capable of reducing a large number of one and two electron acceptors including cytochrome c, ferricyanide, 2,6 dichlorophenolindophenol, menadione, nitroblue tetrazolium and 3-acetylpyridine adenine dinucleotide phosphate (Vermillion and Coon, 1978a).

The uncleaved form of reductase purified from detergent solubilised microsomes was found to have a molecular weight of 76-80 kDa. and to support P450-dependent reactions (Yasukochi and Masters, 1976). In contrast to the P450 superfamily of enzymes only one form of the P450red has ever been isolated from mammals. Southern blot analysis suggests that the reductase is the product of a single copy gene (Porter *et al.*, 1991).

Although the most extensively studied acceptors of electrons from P450red are the cytochrome P450 superfamily of enzymes, P450red has been shown to reduce a variety of other endogenous substrates and artificial compounds. As well as the one and two electron acceptors described above, P450red has been shown to be involved in nitroreduction (Heimbrook and Sartorelli, 1986) and in azoreduction of some compounds (Zbaida and Levine, 1990). P450red has also been shown to reduce mitomycin c (Keyse *et al.*, 1984) and the novel benzotriazine SR4233 (Walton *et al.*, 1992) which can result in the activation of biologically active quinones and the production of reactive oxygen radicals through redox cycling (Kappus, 1986). Thus, levels of P450red may be important in the response to certain types of cancer chemotherapy. Other catalytic mechanisms carried out by the reductase involve the reduction of cytochrome $b_5$ (Enoch and Strittmatter, 1979). A large number of reactions appear to be catalysed by a cytochrome $b_5$ mechanism including fatty acid elongation (Keyse *et al.*, 1979), phospholipid desaturation (Pugh and Kates, 1977) and stearyl CoA desaturation (Shimekata *et al.*, 1972). P450 reductase also appears to be involved in supplying electrons to heme oxygenase (Schahter *et al.*, 1972; Maines, 1988). One product of heme degradation catalysed by the heme oxygenase system is carbon monoxide. Recent work showing the colocalisation of P450red and heme oxygenase along with the rate limiting enzyme in heme biosynthesis (ALS, amino laevulenate synthetase) and the carbon monoxide sensitive guanylyl cyclase in the brain has led to speculation that carbon monoxide produced by this system may be involved in neurotransmission (Verma *et al.*,1993). This speculation was based on *in situ* hybridisation analysis and the similarity between this system and the nitric oxide system (Bredt *et al*, 1991).

After the identification of P450red as the electron donor for P450- mediated reactions, the mechanism of action of this enzyme attracted considerable attention. Interest in the protein also arose because of its unique property of containing two different flavins. Since P450red contains the flavins FMN and FAD as prosthetic groups (Iyanagi and Mason, 1973) it appears yellow in

colour. A useful characteristic of flavins is that their absorption spectra are altered by changes in their reduction state. Therefore, the reduction state can be examined by measuring changes in the visible absorbance range. Each flavin is capable of carrying up to two electrons, therefore P450red can exist in a state containing anywhere between zero and four electrons. Hence, there are nine potential P450red subforms possible. Spectra of all of these subforms have now been obtained by both experimental means and computer modeling (reviewed in Backes, 1993). A variety of these spectra will be discussed and shown in relation to the work presented in Chapter 4 of this thesis.

Many studies in the field of P450red research have come to the conclusion that FAD is the low potential flavin and accepts reducing equivalents from $NADPH^+$ while FMN is the high potential flavin, recieving reducing equivalents from $FADH\cdot$ or $FADH_2$ and transferring them to P450 and cytochrome c or other electron acceptors (Backes, 1993). The FAD site appears to be involved in the reduction of ferricyanide but is not the major site for the reduction of other one electron acceptors (Vermillion and Coon, 1978b). The transfer of electrons between flavin species has been examined in some detail by a laser flash photolysis technique (Bhattacharyya *et al.*, 1991). Rapid intramolecular transfer between flavins was observed. The major function of P450 reductase is to transfer electrons to cytochrome P450, but, because it has the potential of nine different subforms (depending on the number of electrons present) it cannot be treated as a simple electron donor. Each reductase subform will have its own characteristic ability to donate electrons to an acceptor such as P450. Work by Backes and Recker-Backes (1988) suggest that both the 3 and 4 electron containing forms of P450red can rapidly transfer electrons to the P450s CYP1A2 and CYP2B4, whereas the 2 electron containing reductase can donate electrons at approximately 10% of the rate found for the more reduced subforms.

Evidence thus suggests that NADPH cytochrome P450 oxidoreductase has the ability to bind FMN, FAD, NADPH and to interact with membranes and proteins such as cytochrome P450. However, in spite of the importance and complexity of this protein little is understood about the relationship of the function of P450red to its physical structure.

### 1.2.3. Mechanism of cytochrome P450 catalysed reactions.

Stoichiometric studies on the monooxygenation reactions catalysed by P450s have indicated the consumption of one molecule each of NADPH and molecular oxygen resulting in the introduction of one oxygen atom into the substrate molecule (Equation 1). This indicates the supply of two reducing equivalents from NADPH to one P450 molecule during one cycle of the reaction (Guengerich, 1991). Titration of purified P450 with sodium dithionite in the absence of oxygen shows that complete reduction of P450 occurs by one reducing equivalent per mole of heme (Peterson *et al.*, 1977). Therefore, the mechanism of P450-catalysed oxygenation must explain the introduction of a second electron into the P450 in the reaction cycle. P450red has been identified as the physiological donor of electrons for this system (see 1.2.2). Cytochrome $b_5$ may also be involved in the donation of the second electron, although the exact role of this cytochrome in the monooxygenation system has not been fully characterised. It appears that some P450s require cytochrome $b_5$ for efficeint catalysis in reconstituted systems whereas others do not (Schenkman, 1976; Pompon and Coon, 1984).

Cytochrome P450 is present in the endoplasmic reticulum in a 10-25 fold molar excess over P450red (Estabrook *et al.*, 1971; Peterson *et al.*, 1976). However, the functional interaction between these two enzymes appears to be at a ratio of 1:1 (Miwa *et al.*, 1979; Miwa and Lu, 1984). Therefore, only a fraction of the P450 pool can exist in a functional complex with P450red at any one time. Thus, accessibility to P450red may be rate limiting. This could be of importance when a P450 is induced by xenobiotics. Although P450red can also be induced by a variety of xenobiotics it is not usually up-regulated to the same extent as some P450s (Gonzalez, 1989). Two possible mechanisms for the interaction between a P450 and P450red have been proposed (Miwa and Lu, 1984; Wagner *et al.*, 1984). The first proposes that the two enzymes form a stable and catalytically active complex. The second suggests that transient complexes are formed as a result of random collisions occuring from lateral diffusion within the membrane of the endoplasmic reticulum. Although the exact mechanism of interaction has not yet been elucidated, evidence points to there being an electrostatic interaction between P450s and P450red (Berhardt *et al.*, 1984, 1988; Schenkman *et al.*, 1991). However, interactions may differ between the isoforms of P450. It has been demonstrated that specific lysine and arginine residues on P450 (rat CYP1A1) are involved in forming an

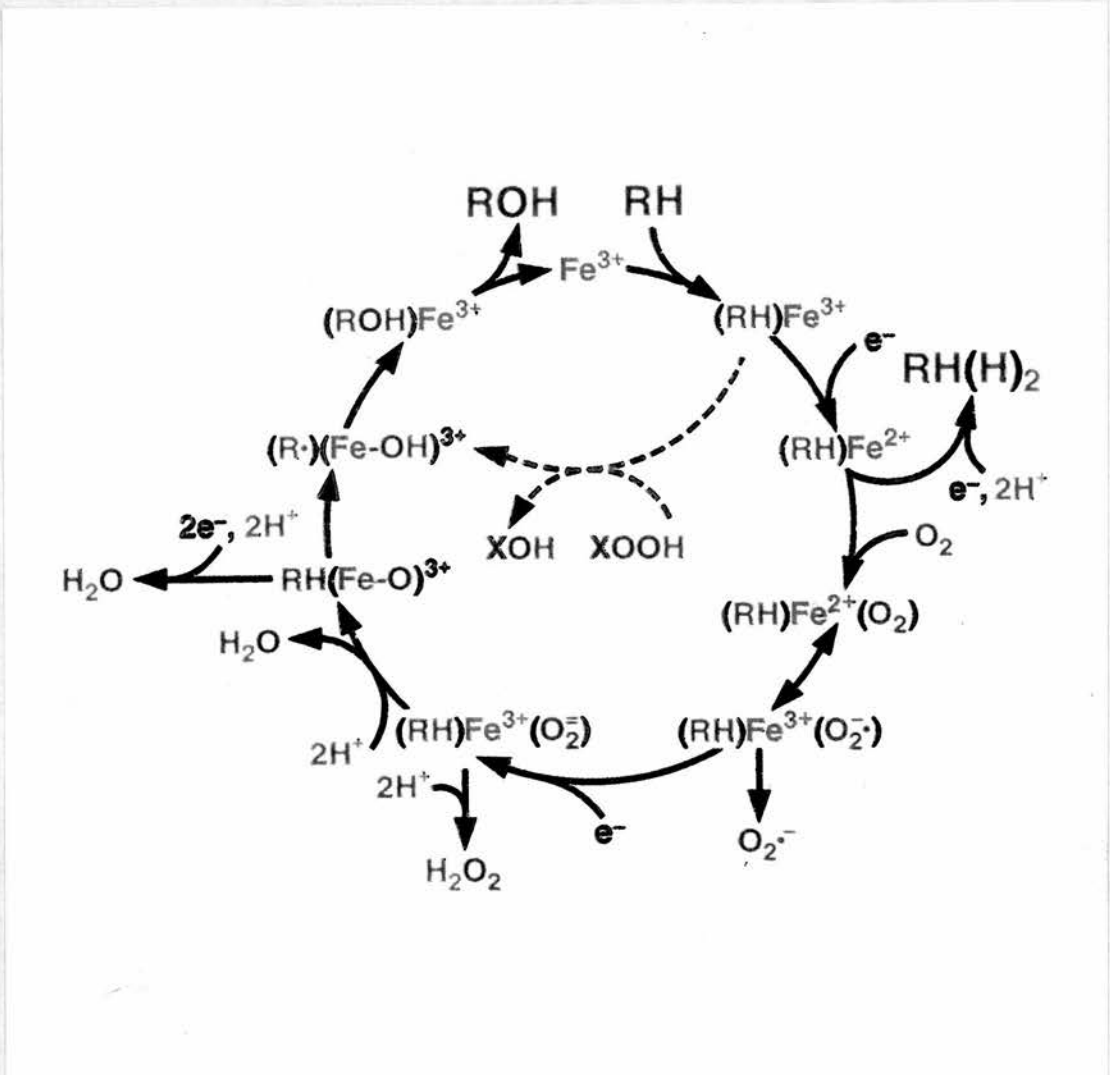



**Figure 1.2. Proposed catalytic cycle for P450-mediated reactions.** (From Porter and Coon, 1991).

electron transfer complex with P450red (Shimizu *et al.*, 1991). Covalent modifications of lysine residues on this protein was also shown to affect interactions with P450red in a reconstituted system. With regard to P450red, Nadler and Strobel (1991) showed in a cross-linking study that a cluster of acidic amino-acids (100-120) on the reductase was involved in the interaction with rat CYP1A1.

The scheme for the mechanism of action of cytochrome P450 is shown in Figure 1.2. The first step in the reaction cycle is substrate binding, which perturbs the spin state equilibrium of the cytochrome and facilitates the uptake of the first electron from P450red. This electron is derived from the two electron donor NADPH and hence the reductase can be thought of as a transducer. The mechanism by which P450red passes reducing equivalents from NADPH through firstly FAD and then FMN onto P450 is a highly complex process which has been studied for over the last twenty years (reviewed by Backes, 1993). Substrates which undergo reduction by P450, rather than oxygenation, such as epoxides, N-oxides, nitro and azo compounds, and lipid hydroperoxides accept two electrons in a step-wise fashion to give $RH(H)_2$. To initiate the oxidative reactions, $O_2$ is bound to the ferrous P450. This intermediate can also be described as the resonance form, $Fe^{3+}(O_2^{-})$, with substrate still present. Transfer of the second electron then occurs with the possible involvement of cytochrome b5 as an additional electron donor in mammalian systems (Schenkman *et al.*, 1976; Pompon and Coon, 1986). The next step is less well understood. It involves splitting of the oxygen-oxygen bond and the uptake of two protons with the generation of an 'activated oxygen' and the release of $H_2O$. Oxygen insertion into the substrate is believed to involve hydrogen abstraction from the substrate and recombination of the resulting transient hydroxyl and carbon radicals to give the product (Porter and Coon, 1991). Dissociation of ROH then restores the P450 to the starting ferric state. Also shown in Figure 1.2 is the mechanism by which a peroxy compound may substitute for $O_2$ and reducing equivalents in what is known as the peroxide shunt.

A schematic representation of P450 redutcase, cytochrome $b_5$ and P450 is shown in Figure 1.3.

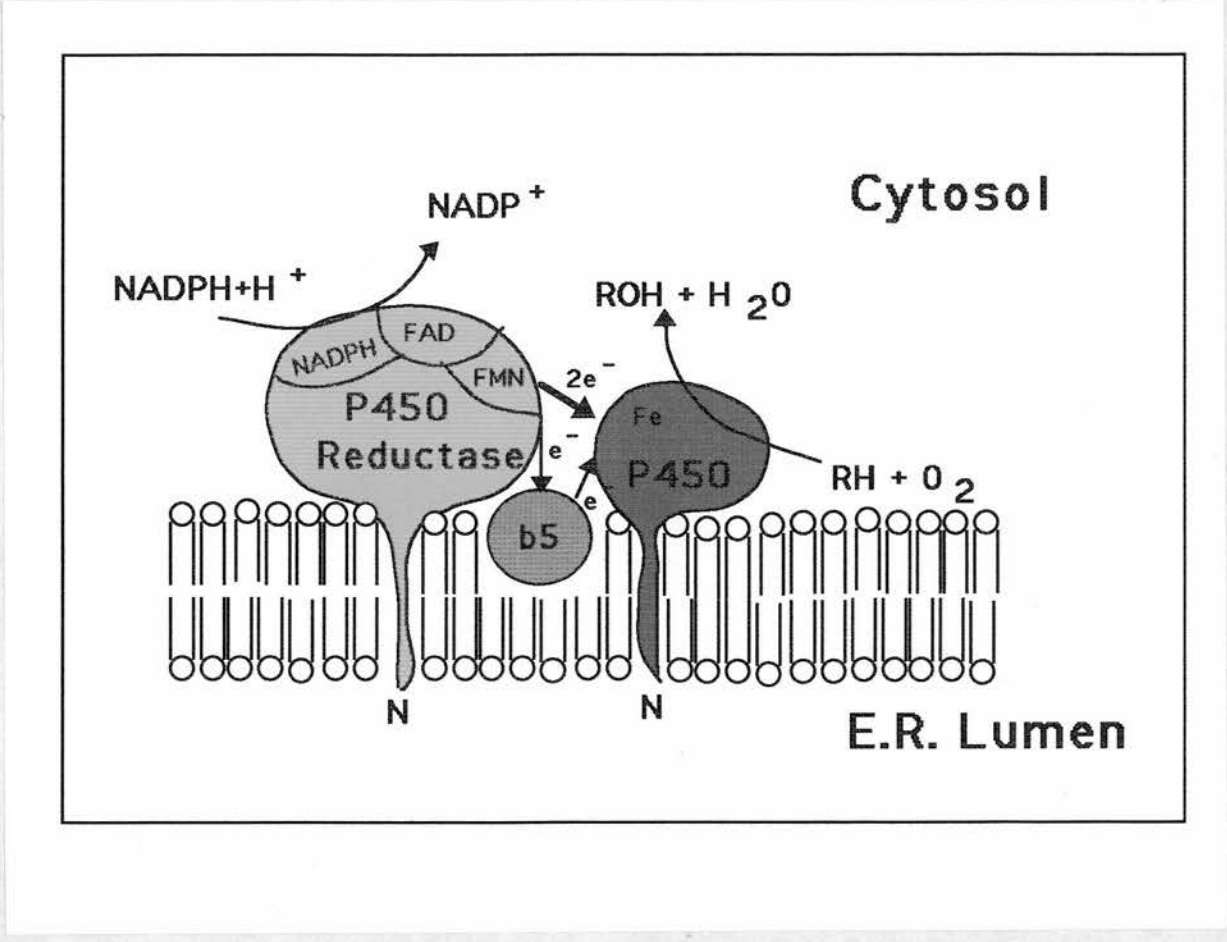



**Figure 1.3. Schematic representation of the P450 monooxygenase system in the endoplasmic reticulum.**

### 1.2.4. UDP-Glucuronosyltransferases.

One of the most important phase II conjugation reactions is that of glucuronide conjugation. Many functional groups have the potential to be glucuronidated including those found on unmodified compounds as well as the products of phase I metabolism. In glucuronidation, the sugar acid D-glucuronic acid is coupled with compounds through -OH, -SH or NH groups to form the β-D-glucopyranosiduronic acids or glucuronides. It is the UDP-glucuronosyl transferases (UDPGTs) that catalyse the transfer of glucuronic acid to the suitable compound to form the glucuronic conjugate. Similar to the cytochrome P450 monooxygenase system, many endogenous compounds serve as substrates for the glucuronidation reaction, including bilirubin, steroid hormones, thyroxine and triiodothyronine The availability of the co-substrate UDPGA is a critical determinant of the glucuronidating capacity of a tissue (Mulder *et al.*, 1990) with the liver usually containing the highest amounts of UDPGA.

UDP-Glucuronic acid + ROH —> RO-glucuronide + UDP (Equation 3)

Like the P450 superfamily of enzymes it is has become clear through the advances in cDNA and gene cloning that the UDPGTs form a large family of isozymes (Burchell *et al.*, 1991). This heterogeneityof UDPGTs had been observed in earlier studies employing enzyme assays, tissue distribution and purification studies (Burchell and Coughtrie, 1989).
Similar again to the P450 superfamily, the UDPGTs are inducible by a variety of xenobiotics including phenobarbital, 3-methylcholanthrene and peroxisome proliferators (Burchell and Coughtrie, 1989).
Analysis of the topology of these 50-56 kDa. glycoproteins (using antibodies, protease and computer predictions) suggests that the transferases are slightly exposed on the cytoplasmic surface. This region is joined by a membrane spanning region to the majority of the protein, including the active site, on the luminal side of the endoplasmic reticulum.

### 1.2.5. Epoxide hydrolases.

Epoxide hydrolases catalyse the formation of dihydrodiols and other glycols from biologically reactive epoxides (Oesch and Daly, 1971). It is the metabolism of compounds by the P450 system that can produce these highly electrophilic epoxides which are capable of modifying cellular macromolecules. The epoxide hydrolase converts these intermediates into the chemically less reactive dihydrodiols. There are several forms of epoxide hydrolase including both cytosolic and microsomal species. The major form of microsomal epoxide hydrolase (mEH) hydrates a wide range of substrates including epoxides of cyclic compounds. The dihydrodiols that are produced may then be conjugated by a phase II system and ultimately excreted. However, some polycyclic aromatic diols can serve as substrates for P450s which can result in the production of highly reactive potentially carcinogenic diol epoxides. Therfore the epoxide hydrolases play a central role in both the detoxication of xenobiotics and in the activation of metabolites to more potent carcinogens. This pathway of detoxication/activation is shown in Figure 1.4. Purified preparations of microsomal epoxide hydrolase exhibit molecualr weights of approximately 48-54 kDa. This is in agreement with the deduced sizes predicted from the cDNAs obtained from a variety of species including man. Compared to other species, human liver microsomes contain relatively high levels of epoxide hydrolase activity with substantial variation in the enzyme activity within the human population (Kapitulnik 1977).

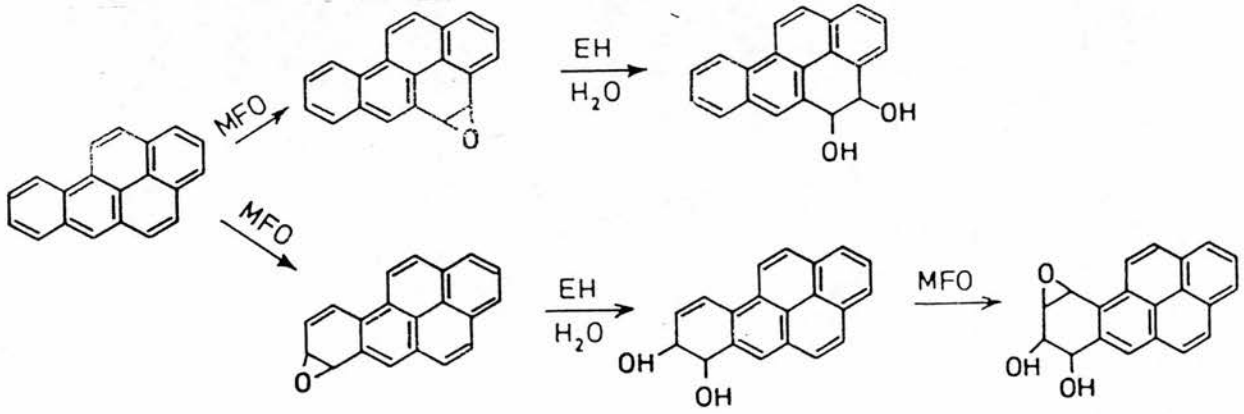



**Figure 1.4.** Epoxide formation and metabolism. Scheme for the metabolism of the polycyclic aromatic hydrocarbon, benzo(a)pyrene resulting in the formation of of epoxides , diols and diol-epoxides.

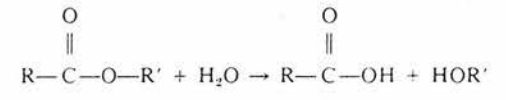
### 1.2.6 Flavin Containing Monooxygenases.

The flavoprotein that catalyses NADPH and oxygen-dependent oxidation of a wide variety of xenobiotics was previously termed the microsomal mixed function amine oxidase and is now known as the flavin containing monooxygenase (Jakoby and Zeigler 1990). With few exceptions, virtually all xenobiotics bearing amine, hydrazine, thiol, disulphide, thiocarbamide, thioamide, thio acid, dithiocarbamide or phosphine groups serve as substrates. Hence this broad substrate specificity resesmbles that of the P450 monooxygenase system The flavin containing monooxygenase is a polymeric protein exhibiting a monomeric molecular weight of 65 kDa. and contains one mole of FAD per mole of protein (Ziegler and Mitchell, 1972; Poulsen and Ziegler, 1979). The flavin containing monooxygenase is the only mammalian flavoprotein hydroxylase known although many bacterial examples are known. Using N, N'-dimethylaniline as a representative substrate, the flavin containing monooxygenase system catalyses the following reaction (Equation 3).

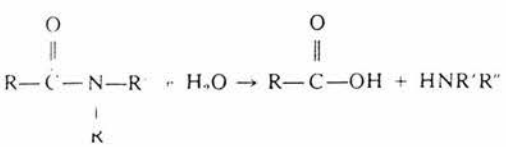
$$NADPH + H^+ + O_2 + C_6H_5\text{-}N(CH_3)_2 \longrightarrow NADP^+ + H_2O + C_6H_5\text{-}N(CH_3)_2\text{->}O$$

### 1.2.7. Carboxylesterases

The mammalian carboxylesterases are a family of non-specific hydrolases found in abundance in the endoplasmic reticulum of the liver (Hosokawa *et al.*, 1989). These enzymes catalyse the hydrolysis of many xenobiotics and therapeutic drugs containing ester and amide bonds (Heymann, 1982). However, in many cases hydrolysis of these bonds is only a minor detoxication pathway. The normal chemical mechanism for the hydrolysis of ester and amide bonds are very similar and shown in Equation 4 below.



Carboxylester hydrolysis



Carboxyamide hydrolysis

The liver microsomal carboxylesterases have been shown to be involved in the hydrolysis of ester bonds in narcotics, local anaesthetics and analgesics. Esterification of drugs to form prodrug esters may result in improved properties as compared to the parent compound. By esterification with small aliphatic or aromatic carboxylic acids the polarity of the drug is reduced. This promotes absorption through the intestinal wall. For example, inactive esters of the centrally acting drug oxazepam are bioactivated by hepatic carboxylesterase (Maksey *et al.*, 1977). Prodrugs can also be used to prolong the action of drugs. Hydrolysis of peptide bonds in prodrugs has been used successfully to increase the selectivity of anticancer drugs (Carl *et al.*, 1980). The microsomal carboxylesterases will be discussed in more detail in the introduction to chapter 5 where the purification and characterisation of a human microsomal carboxylesterase will be discussed.

## 1.3 Summary

This introduction has served to highlight some important aspects of drug metabolism and to give an overview of some of the enzyme systems involved in the metabolism of exogenous compounds found in the endoplasmic reticulum. Particular emphasis was placed on the P450 monooxygenase system as studies on NADPH cytochrome P450 oxidoreductase are the major theme of the work presented within this thesis. The use of a bacterial expression system to study the role of this enzyme in the bioactivation of redox cycling compounds will be the focus of work in chapter 3. Structural and functional studies of NADPH cytochrome P450 oxidoreductase are dealt with in chapter 4, where the use of a bacterial expression system was used to express and purify a variety of domains of structural importance. Chapter 5 deals with the purification and characteristion of a human hepatic microsomal carboxylesterase. The role of this enzyme in an immune mediated adverse drug reaction, halothane hepatitis, will be considered.

## Chapter 2: Materials and Methods.

### 2.1 Bacterial strains, growth media, antibiotics and transformation of bacteria.

#### 2.1.1 Bacterial Strains.

| | |
|---|---|
| *E. coli*: JM109 | (*rec* A1, *sup* E44, *end* A1, *hsd* R17, *gyr* A96, *rel* A1, *thi* D (*lac-pro* AB) ) F' [*tra*D36 *pro*AB$^+$*lacI$^q$* *lacZ* DM15] |
| NM522 | *Sup E, thi*-1 D (*lac-pro* AB), D(*hsd SM M-crB*)5 ($n_k$-$m_k$) |

These strains were used as general hosts for recombinant manipulations and for heterologous expression.

| | |
|---|---|
| *E. coli*: BL21(DE3) | *hsd* S *gal* (lcI *ts* 857 *ind* 1 *Sam* 7 *nin* 5 *lac* UV5-T7 gene 1) pLysS. |

This strain was used for heterologous expression studies when using the pET15b system. Note, the plasmid pLysS contains a copy of T7 lysozyme under the control of *laq I$^q$* and is selected for using chloramphenicol.

*S. typhimurium:* The strains TA1538 and LR5000, as described by Ames *et al.,* (1979), were used as hosts for heterologous expression.

#### 2.1.2 Bacterial culture media.

| | |
|---|---|
| L.B.-broth/litre: | 10 g bactotryptone (Difco), 5 g yeast extract (Difco), 5 g NaCl. |
| L.B.-agar/litre: | As for L.B.-broth with an additional 15 g/litre of agar (Difco). |
| Minimal agar/litre: | 200 ml 5x M9 salts (64 g $NaHPO_4.7H_20$, 15 g $KH_2PO_4$, 2.5 g NaCl, 5 g $NH_4Cl$, 20 ml 20% glucose (w/v)), supplemented with maltose to 0.2% (w/v) and |

$MgSO_4$ to 0.1 mM. This agar was used to ensure retention of the F' episome in bacterial strains.

### 2.1.3 Antibiotics.

Ampicillin: Stock of 50 mg/ml prepared in distilled water, used at a working concentration of 50 μg/ml in both media and agar plates.

Chloramphenicol: Stock of 34 mg/ml prepared in ethanol, used at a working concentration of 34 μg/ml in both media and agar plates.

### 2.1.4 Bacterial transformation using calcium chloride.

0.5 ml of an overnight culture of *E.coli* was added to 20 ml of L.B. broth and allowed to grow with shaking until the culture reached an $O.D._{600}$ of 0.5-0.6. Cells were pelleted by centrifugation at 6,000 g, 4°C for 5 minutes, resuspended in 10 ml of 100 mM calcium chloride and kept on ice for 1 hour. The cells were pelleted again in an identical manner and resuspended in 2 ml of 100 mM calcium chloride. Cells were stored at 4°C and used within 2 days of preparation.
Ligation mix or plasmid DNA (0.01 μg-1 μg) was added to 200 μl of competent cells and left on ice for 1 hour. The cells were then heat shocked at 42°C for 90 seconds and added to 500 μl of prewarmed L.B.-broth. The cells were pre-expressed at 37°C for 1 hour to allow for the expression of resistance proteins. The cells were then pelleted, 6,000 g for 5 mins, resuspended in 200 μl of fresh L.B. broth and applied to an L.B. agar plate, containing the appropriate antibiotic(s), inverted and incubated overnight at 37°C .

### 2.1.5 Transformation of bacteria by electroporation.

Since calcium chloride treatment (2.1.4) does not generate a high level of transformation competent cells in *S.typhimurium*, these bacteria were transformed by electroporation. This was carried out by mixing 0.1 μg of plasmid DNA with 500 μl of bacteria, $O.D._{600}$ = 0.6, and electroporating them at 40 μF, 0.2 kV using an IBI electroporator. The cells were pre-expressed at

37°C for 1 hour to allow for the expression of resistance proteins. The cells were then pelleted, 6,000 g for 5 mins, resuspended in 200 µl of fresh L.B. broth and applied to an L.B. agar plate, containing the ampicillin at 50 µg/ml, inverted and incubated overnight at 37°C.

## 2.2 DNA isolation and analysis.

### 2.2.1 Plasmids used in this study.

| Plasmid | Use |
|---|---|
| pTZ19R (Pharmacia) | General cloning |
| pCITE2a (Novagen) | General cloning;has a very large polylinker |
| pKK223-3 (Pharmacia) | Bacterial expression |
| pOR263 (Shen *et al.*, 1989) | Bacterial expression |
| pET15b (Novagen) | Bacterial expression |

### 2.2.2 Plasmid preparations.

### 2.2.2a DNA isolation from small scale (5 ml) bacterial cultures.

Bacteria from an overnight culture were harvested by centrifugation at 6,000 g for 10 minutes and resuspended in 100 µl of 10 mM Tris.HCl, 0.1 mM EDTA buffer, pH 8.0. The cells were lysed by the addition of 200 µl of 0.2 M NaOH, 1% SDS (w/v) and left at room temperature for 5 minutes. To the lysed bacteria 40 µl of 3 M potassium acetate, pH 4.8, was added and left at room temerature for 2 minutes before centrifugation, 13,000 g for 15 minutes, 4°C, to remove genomic DNA and cell debris. RNA was precipitated from the supernatent fraction by the addition of 450 µl of 5 M LiCl and storage at -20°C for at least 30 minutes. The precipitated RNA was removed by centrifugation, 13,000 g for 15 minutes, 4°C, before precipitating the plasmid DNA with 1.5 volumes of isopropanol. After being left at room temperature for at least 5 minutes, the DNA was pelleted by centrifugation, 13,000 g for 10 minutes, room temperature, and then washed with 70% ethanol before being dried in a vacuum oven at 30°C. The resulting plasmid DNA was dissolved in 25-50 µl 10 mM Tris.HCl, 0.1 mM EDTA buffer, pH 8.0. 5 µl of RNAse A (2 mg/ml) was added to the DNA and incubated at 37°C for 15-30 minutes.

### 2.2.2b DNA isolation from medium scale (up to 100 ml) and large scale (above 100 ml) bacterial cultures.

Qiagen DNA affinity columns (Qiagen Inc.) were used in the isolation of plasmid DNA from medium and large scale bacterial cultures. Steps in the procedure were scaled up accordingly from the protocol described above (2.2.2a) up to the addition of 3 M potassium acetate (pH 4.8). At this point the sample was immediately centrifuged for 30 minutes at 30,000 g. The supernatent, containing the plasmid DNA, was applied to an appropriately sized Qiagen column which had been previously equilibrated with buffer QBT (750 mM NaCl, 50 mM MOPS, 15% (v/v) ethanol, 0.15% (v/v) Triton X-100, pH 7.0) and allowed to enter the resin under gravity. The column was washed with 3 column volumes of buffer QC (1 M NaCl, 50 mM MOPS, 15% (v/v) ethanol, pH 7.0) during which the plasmid DNA remains bound to the resin. The plasmid DNA was eluted from the resin with 1.5 column volumes of high pH buffer QF (1.25 M NaCl, 50 mM MOPS, 15% (v/v) ethanol, pH 8.2). The plasmid DNA was precipitated from the eluant by the addition of 0.7 volumes of isopropanol and collected by centrifugation at 30,000 g for 30 minutes. The DNA pellet was resuspended in 50 µl of 10 mM Tris.HCl, 0.1 mM EDTA, pH 8.0 per 100 ml of initial culture.

### 2.2.3 DNA concentration estimation.

Spectrophotometric readings of a known dilution of DNA samples were taken at 260 nm and 280 nm. An $A_{260}$ reading of 1 corresponds to approximately 50 µg/ml for double stranded DNA. An estimation of sample purity is provided by the ratio of the absorbance values at 260 nm and 280 nm, with pure DNA having a value of 1.8.

### 2.2.4 Restriction endonuclease analysis of DNA samples.

Restriction endonucleases were obtained from Boehringer Mannheim (BCL), New England Biolabs, Bethesda Research Laboratories (BRL) or Amersham International (Amersham). Digestions were carried out using the manufacturer's recommended buffer and conditions.

### 2.2.5 Agarose gel electrophoresis of DNA.

DNA samples were analysed using horizontal gel electrophoresis tanks (BRL). Agarose gels were prepared from electrophoresis grade (Ultra Pure, BRL) agarose for both diagnostic and preparative gels. Agarose was used at a variable concentration between 1 and 2% (w/v) in TAE buffer (0.04 M Tris-acetate, 1 mM EDTA, pH 8.0). 0.2 volumes of gel-loading dye (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 15% (w/v) ficoll, in water) was added to DNA samples before applying them to the gel. Following electrophoresis, the DNA samples were stained with ethidium bromide by the addition of ethidium bromide to a concentration of 0.5 μg/ml in water. The gel was then washed extensively with distilled water and the DNA visualised using a short wavelength trans-illuminator.

### 2.2.6 DNA fragment isolation and preparation for subcloning.

The DNA fragment ("insert DNA") to be subcloned, whether derived from a plasmid or the polymerase chain reaction (PCR), was separated from other DNA material on a 1- 2% agarose gel depending on the size of the fragment. The DNA of interest was dissected from the body of the ethidium bromide stained gel using a scalpel. The insert DNA was separated from the agarose using either Geneclean (Bio 101 inc.) glass bead solution or GlassMax (BRL) glass matrix spin columns according to the manufacturer's instructions. If the insert DNA required further modification prior to subcloning, e.g. 'end-filling' or restriction endonuclease digestion, then these manipulations were performed. Following these modifications the DNA was purified from any of the contaminating proteins by using the Geneclean or Glassmax systems, as discusssed above. The DNA was resuspended in a volume of 10-20 μl (10 mM Tris.HCl, 0.1 mM EDTA, pH 8.0).

### 2.2.7 Vector preparation for the receipt of DNA inserts.

The vector into which the DNA insert was to be subcloned was digested with the appropriate restriction endonucleases and subjected to any further modifications such as "end-filling" or dephosphorylation. Following these manipulations the vector was purified from any contaminating proteins by using the Geneclean or Glassmax systems (2.2.6) and resuspended in 10 mM

Tris.HCl, 0.1 mM EDTA, pH 8.0. The DNA concentration was then estimated (2.2.3).

### 2.2.8 "End-filling" recessed 3' terminals of PCR products using the "Klenow" fragment.

In order to generate DNA molecules with non-recessed 3' termini from PCR products the recessed terminii can be filled in by the action of the large fragment of *E. coli* DNA polymerase I, termed the "Klenow" fragment (BRL). 1-10 µg of DNA was mixed with 50 mM Tris.HCl, pH 7.5 10 mM $MgCl_2$, 0.25 mM each dNTP (Pharmacia) and 1 unit of Klenow fragment in a final volume of 25 µl and incubated at 37°C for 30 minutes. The DNA was purified from the enzyme and unincorporated dNTPs using Geneclean (2.2.6) and resuspended in 20 µl of 10 mM Tris.HCl, 0.1 mM EDTA, pH 8.0.

### 2.2.9 Dephosphorylation of 5' terminal phosphates.

Vectors linearised using a single restriction endonuclease or with two restriction endonucleases, one of which may be inefficient in its cutting (*e.g. Nde* I) may recircularise on ligation. To reduce this possibility 5' terminal phosphates were removed from digested vector DNA using calf intestinal phosphatase (CIP) (Promega). 1-5 µg of DNA was resuspended in 40 µl of 10 mM Tris.HCl ,pH 8.0 and 5 µl of 10x CIP buffer (0.5 mM Tris.HCl, pH 9.0, 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 10 mM spermidine). To this, 0.5 units of CIP was added and the sample incubated at 37°C for 30 minutes followed by the addition of a second 0.5 unit aliquot of CIP for a further 30 minutes at 37°C. The DNA was then purified from any contaminants using Geneclean (2.2.6) and resuspended in 10-20 µl of 10 mM Tris.HCl, 0.1 mM EDTA, pH 8.0.

### 2.2.10 Ligation of the prepared insert and vector DNA.

For ligation of DNA fragments and vectors with cohesive terminals, blunt ends or with both, 100-200 ng of vector DNA and a 3 fold excess of insert DNA were mixed to a final volume of 7.5 µl. To this was added 1 µl of 10x ligation buffer (200 mM Tris.HCl, pH 7.6, 50 mM $MgCl_2$, 50 mM dithiothreitol), 1 µl of 5 mM ATP and 1 unit of T4 DNA-ligase (BCL). The reaction was allowed to proceed for no longer than 16 hours at 12°C.

### 2.2.11 Screening of bacterial colonies transformed with recombinant plasmid constructs.

#### 2.2.11a α-complementation screening of bacterial colonies.

The vector pTZ19R used in this study carries a short segment of DNA that contains the regulatory sequence and first 146 amino acids of the *E.coli lac Z* gene encoding the β-galactosidase enzyme. On transformation of the parent plasmid into certain host strains, which produce the C- terminus of the β-galactosidase enzyme encoded on the F' episome, the two fragments can interact and produce an active protein (Ullman *et al.*, 1967). This process is termed α-complementation. Bacteria transformed with the parent vector form blue colonies in the presence of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) after induction of the *lac Z* gene fragments by IPTG addition. However, insertion of a recombinant fragment (the "insert") into the cloning site of the vector leads to disruption of the *lacZ* N-terminal fragment resulting in colonies that appear white in the presence of X-gal or IPTG.
Bacteria transformed with pTZ19R, which allows α-complementation to be performed, were plated onto L.B. agar/ampicillin plates which were prespread with 40 µl of X-gal (20mg/ml in dimethylformamide) and 40 µl of IPTG (20 mg/ml in water).

#### 2.2.11b Screening of bacterial transformants by colony hybridisation.

A modification of the method of Grunstein and Hogness (1975) was used to screen for bacterial transformants. Transformed colonies were picked using sterile toothpicks and duplicate colonies streaked onto gridded nitrocellulose membranes that were placed on L.B. agar plates containing ampicillin. Plates were inverted and incubated at 37°C overnight. One of these plates was stored at 4°C (the 'master" plate). The second filter was removed from its plate and placed colony side-up on Whatman 3MM paper soaked in 10% SDS (w/v) for 3 minutes, denatured for 5 minutes (0.5 M NaCl, 1.5 M NaOH) and neutralised for 5 minutes (0.5 M Tris.HCl, pH 7.5, 1.5 M NaCl). The filter was air dried at room temperature and baked in a vacuum oven at 80°C for 2 hours. The bacterial debris was removed from the filter by washing in 50 mM

Tris.HCl, pH 8.0, 1.5 M NaCl, 1 mM EDTA and 1% SDS (w/v) in a shaking water bath at 42°C for 1 hour. Filters were then prehybridised and hybridised using a radiolabelled insert as a probe (see 2.2.12 and 2.2.13 below). Recombinant colonies that hybridised with the probe were picked and grown from the duplicate colony on the retained master filter.

### 2.2.12 Random prime labelling of double-strand derived DNA probes.

Double stranded DNA was labelled by the random priming method of Feinberg and Vogelstein (1983). The DNA insert was heat denatured in the presence of a molar excess of random hexanucleotide primers. Radioactive nucleotides were added into the probe DNA following primer extension by the large fragment of *E.coli* DNA polymerase I (Klenow fragment). The above was carried out as follows; 50 ng of the DNA to be labelled was denatured at 100°C for 3 minutes and mixed with the following components:

| | |
|---|---|
| OLB (see below) | 3µl |
| BSA (10mg/ml, Sigma) | 2µl |
| Klenow fragment | 2 units |
| [$\alpha$-$^{32}$P] dCTP sp.activity> 3,000 Ci/mmole; 10Ci/ml | 3µl |
| $ddH_20$ | to 100µl |

OLB comprised of a mixture of the solutions A,B and C in a ratio of 2:5:3

| | | |
|---|---|---|
| A: | 2M Tris.HCl, pH 8.0 | 625 µl |
| | 5M MgCl2 | 25 µl |
| | $DDH_2O$ | 350 µl |
| | 2-mercaptoethanol | 10 µl |
| | 100mM dTTP, dCTP, dGTP, dATP | 5 µl of each |
| | in 3mM Tris.HCl, pH8.0, 0.2M EDTA (Pharmacia) | |

B: 2M Hepes (pH 6.6)

C: Hexadeoxyribonucleotides in 3 mM Tris.HCl (pH 7.0), 0.2 mM EDTA to a concentration of 90 O.D.units/ml (Pharmacia)

### 2.2.13 Establishing the efficiency of radiolabel incorporation into double strand DNA derived probes.

1 µl of the labelled probe mixture was spotted onto Whatman DE-81 filter paper. The filter is positively charged and binds oligonucleotides and double stranded DNA whereas unincorporated nucleotides are bound less strongly. The nucleotides can be separated from the larger DNA species chromatographically using 0.3 M ammonium formate (pH 8.0). By exposing the resultant filter to X-ray film an assessment of the relative levels of incorporated to unincorporated radiolabelled nucleotides was obtained. An estimated efficiency of greater than 80% was routinely obtained.

### 2.2.14 Hybridisation of radiolabelled DNA probes to membrane bound DNA.

Membrane bound DNA samples were prehybridised for at least 3 hours at 65°C in glass tubes in a rotisserie oven (Techne).
Prehybridisation solution was prepared in $DDH_2O$ as follows:

6X SSC
2X Denhardt's solution
0.1% SDS (w/v)
0.1% Sodium pyrophosphate (w/v)

| | |
|---|---|
| 20X SSC, pH 7.0/ litre: | 175.3 g NaCl, 88.2 g sodium citrate. |
| 50X Denhardt's solution | 10 g each of BSA (Sigma), polyvinylpyrollidine (Sigma), Ficoll-400 (Sigma) made up in $DDH_2O$. |

After prehybridisation the radiolabelled DNA probe was denatured at 100°C for 3 minutes and added to fresh prehybridisation solution. Hybridisation took place at 65°C overnight.
After hybridisation the filters were washed with four changes of 2X SSC and 0.1% SDS at room temperature for 5 minutes. The membranes were then washed in 1XSSC and 0.1% SDS twice for 90 minutes at 65°C. The filters were air-dried on 3MM paper, covered with clear plastic film (Saran-wrap) and exposed to X-ray film in a cassette for 30 minutes at room temperature.

### 2.2.14 Sequencing of double stranded DNA.

Double stranded (ds) DNA templates were sequenced using a modified version of the chain termination method (Sanger *et al.*, 1977) using the Sequenase enzyme (USB), a genetic varient of the T7 DNA polymerase lacking 3'-5' exonuclease activity. DNA templates were prepared using Qiagen columns (2.2.2b). Template denaturation and primer annealing were carried out as follows; to 10 µg of dsDNA, in a final volume of 8 µl, 1 µl of primer (10 ng/ml) and 1µl of 1M NaOH were added and the mixture heated for 10 minutes at 68°C. The sample was neutralised by the addition of 4 µl of TDMN (3.2g TES (Sigma), 0.5ml chloroform, 0.368g DTT, 4 ml 1M MgCl, 2 ml 5 M NaCl, $DDH_2O$ to 50 ml, pH 1.6). The sample was left to cool to room temperature over a period of 10 minutes.
Labelled DNA was generated from the annealed primer in the following extending reaction for 5 minutes at room temperature.

| | |
|---|---|
| Annealed template/ primer mix | 10 µl |
| 0.1 M dithiothreitol | 1 µl |
| 5X Sequenase labelling mix | 0.4 µl |
| [α-$^{35}$S]dATP sp. activity 1000 Ci/mmol, 10mCi/ml | 1 µl |
| Sequenase (diluted 1:5 with enzyme dilution buffer) | 2 µl |

5X labelling mix: 7.5 mM each of dCTP, dGTP, dTTP
Sequenase dilution buffer: 10 mM Tris.Cl, pH 7.5, 5 mM dithiothreitol
0.5 mg/ml BSA.

4 µl aliquots of the extension reaction were terminated following the addition to separate tubes containing 2.5 µl of 8 mM each ddNTP. The termination reaction was incubated for 10 minutes at 37°C and was stoppped by the addition of 4 µl of loading buffer (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF). The samples were heated at 100°C for 2 minutes and resolved on a 6% acrylamide, 7 M urea (Ultrapure), 1X TBE gel. Following electrophoresis the gel was fixed in 10% (v/v) acetic

acid, 10% (v/v) methanol and dried for 2 hours under vacuum at 80°C prior to exposure to X-ray film overnight.

### 2.2.16 The polymerase chain reaction (PCR).

The following reactions were carried out to amplify specific regions of DNA. Details on regions that were amplified will be given at the appropriate point in the text.

| | | |
|---|---|---|
| Template DNA | | 1 µl (1-10ng) |
| dNTPs (2.5 mM of each dNTP) | | 8 µl |
| Oligonucleotide primers (0.5 µg/ml) | | 1 µl each |
| 10X reaction buffer | | 10 µl |
| *Pfu* DNA dependent DNA polymerase | | 1 unit |
| DDH20 | (Stratagene) | to 100 µl |

10X reaction buffer: 10mM Tris.HCl, pH8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin.

The mixture was overlaid with 50 µl of white mineral oil (Sigma) to prevent evaporation before being subjected to the following reactions in a Hybaid Omnigene thermal cycler.

| | | |
|---|---|---|
| Mixing, 92°C | 5 minutes | (1 cycle) |
| Denaturing, 92°C | 30 seconds | (30 cycles) |
| Annealing, 50°C | 60 seconds | (30 cycles) |
| Extending, 72°C | see below | (30 cycles) |

The length of the extension step was varied according to the expected length of the amplified product. An extension time of 1 minute per 1kb was generally used (Innis and Gelfand, 1990).

### 2.2.17 Computer analysis of DNA and amino acid sequence data.

Sequence data was analysed for restriction endonuclease sites, protease sites and for homology with other proteins using the 'Map', 'Pepmap' and 'Bestfit' programs of the GCG package respectively (Devereux *et al.* 1984). Multiple

sequence alignments were carried out using the 'Pileup' and 'Prettybox' programs of the GCG package. Isoelectric point determination was achieved using the 'Isoelectric' program from the GCG package

## 2.3 Biochemical Techniques.

### 2.3.1 Protein concentration determination.

The protein concentration of samples to be analysed was determined by the method of Lowry *et al.* (1951). A range of BSA concentrations, 0-150mg/ml, was used to generate a standard curve each time the assay was performed. The sample to be analysed was mixed 1:40 or 1:200 (v/v) in 0.1M NaOH. To this was added 5 volumes of freshly prepared alkaline carbonate solution (490ml 70 mM $Na_2CO_3$, 40mM NaOH, 5 ml 40 mM $CuSO_4$ and 5ml 71 mM potassium tartrate per 500ml). This solution was then incubated for 10 minutes at room temperature. 0.1 volumes of Folin Ciocalteau reagent was added and the sample incubated for 1 hour at room temperature. Absorbance values at 600 nm were determined using a Shimadzu U.V. 160 spectrophotometer. The standard protein concentration values were determined in duplicate and the sample values in triplicate.

### 2.3.2 Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of protein samples.

Protein samples were prepared for SDS-PAGE at 2X the required concentration and diluted 1:1 with 2X boiling mix (50 mM Tris.HCl, pH 6.8, 2% (w/v) SDS, 5% (w/v) β-mercaptoethanol, 10% (w/v) glycerol, 0.05% (w/v) bromophenol blue) and boiled for 5 minutes.
SDS-PAGE was carried out according to the method of Laemmli (1970). For detection of P450 reductase and carboxylesterase samples were separated on 9% (w/v) polyacrylamide gels (8.7% acrylamide, 0.3% *N, N'*-methylene-bis-acrylamide) in 0.375 M Tris.HCl (pH 8.8), 0.15% (w/v) SDS. Ammonium persulphate (AMPS, 3% (w/v)) and *N, N, N', N'*-tetra-methyl ethylenediamine (TEMED) were added to 0.05% (v/v). For detection of lower molecular weight proteins such as the domains of P450 reductase and cytochrome c, samples were separated on 12% or 15% (w/v) polyacrylamide gels. A stacking gel was prepared (4.5% (w/v) acrylamide, 0.125 M Tris.HCl (pH 6.8), 0.125% SDS, 0.05%

each of TEMED and AMPS) and poured on top of the polymerised separating gel. The gels were prepared in BioRad Protean 2 or BioRad Mini-Protean verical electrophoresis equipment. Protein samples were subjected to electrophoresis using electrode buffer (0.05 M Tris HCl, pH 8.3, 0.05 M glycine, 0.1 % SDS) at 60 mA through the stacking gel and 30 mA through the separating gel. The apparatus was water cooled. After electrophoresis the gels were removed and either stained with coomassie blue (2.3.3) or subjected to Western blot analysis (2.3.4). Molecular weight standards were obtained from Sigma. For 9% gels the following standards were used (SDS-6H): carbonic anhydrase, 29 kDa.; egg albumin, 45 kDa; bovine plasma albumin, 66 kDa; phophorylase b, 97.4 kDa; β-galactosidase; 116 kDa; myosin, 205 kDa. For 12% gels the following standards were used (SDS-6): lysozyme, 14.3 kDa; β-lactoglobulin, 18.4 kDa.; trypsinogen, 24 kDa.; pepsin, 34.7 kDa; egg albumin, 45 kDa.; bovine plasma albumin, 66 kDa. For 15% gels the following standards were used (Boehringer Mannheim Biochemica, 1317 474): Glutamate dehydrogenase, 55 kDa; aldolase, 39.2 kDa; triosephospate isomerase, 27 kDa., trypsin inhibitor, 20 kDa., lysozyme, 14.3 kDa.
The Pharmacia Phast System was also used for SDS-PAGE and isoelectric focusing of the carboxylesterase. Precast gels were used and electrophoresis carried out according to the manufacturer's instructions.

### 2.3.3 Coomassie blue staining of polyacrlyamide protein gels.

Gels were stained and fixed in 0.25% w/v Coomassie blue R, 10% acetic acid, 40% methanol for up to 2 hours. Destaining was performed in 20% methanol, 7.5 % acetic acid.

### 2.3.4 Western blot analysis of SDS-PAGE separated protein samples.

Western blot analysis was performed by a modification of the method of Towbin *et al.* (1979). SDS-PAGE resolved protein was transferred using a BioRad Protean II transblot cell onto 0.45 mm nitrocellulose filters ( Schleicher and Schuell) in transblot buffer (20 mM disodium orthophosphate, 20% (v/v) methanol) for 15 hours at 250 mA. After protein transfer the filter was stained with Ponceau S (0.01%) w/v to check for efficiency of transfer. The filter was washed in TBST (50 mM Tris.HCl, pH7.9, 0.15 M NaCl, 0.05% Tween 20) for 2X 10 minutes. Following this the filter was blocked in 5% (w/v) low fat

powdered milk (Marvel, Cadbury's) in TBST for 1 hour. After blocking and washing the filter for 3X 15 minute periods in TBST, the first antibody, raised against the protein of interest, was added at a dilution of 1:1000 (in TBST) and left for 1 hour. The first antibody was removed and the filter washed for 4X 15 minute periods in TBST before addition of the second antibody at a dilution of 1:1000 (in TBST). Since the first antibodies used in this study were raised in rabbits (2.3.6) the second antisera used was donkey anti-rabbit IgG. After washing (4X 15 minutes in TBST) the immunoreactive protein antibody complexes were probed in 0.19 MBq of $^{125}$I-conjugated protein A in 50 ml of TBST for 45 minutes followed by extensive washing in TBST to remove non-specifically bound radioactivty. The filters were then exposed to X-ray film.
Some modifications on the above procedure were employed when looking for autoantibodies in patients' sera. The method employed has been described previously (Kenna *et al*, 1987). The basic differences were that instead of using Marvel to block the nitrocellulose filter 5% w/v casein (Hammerstan grade, BDH) in TBST was used. The filters were blocked overnight. Also, the washing procedures employed TBST with 0.5% w/v casien. The human serum was diluted 1:100 while the secondary antisera employed was a goat anti-human IgG (diluted 1:1000 in TBST).

### 2.3.5 Transfer of protein to polyvinyldifluoride membranes

Protein samples for solid-phase sequence analysis (Matsuidara, 1987) were transferred from standard polyacrylamide gels (2.3.3) after electrophoresis onto polyvinyldifluoride (PVDF) membranes. Solid phase N-terminal sequence analysis was kindly carried out by Dr. Darryl Pappin, I.C.R.F., London, using a Milligen 6600 solid phase sequencer (Pappin *et al.*, 1990).
SDS-PAGE resolved protein was transferred, using a BioRad Protean II transblot cell, onto methanol washed PVDF (Immobilon, Millipore) in transblot buffer (10 mM 3-cyclohexylamino-1-propanoic acid, pH 11, 10% (v/v) methanol) for 15 hours at 250mA.

### 2.3.5 Development of rabbit anti-serum.

Polyclonal anti-serum against human P450 reductase and human carboxylesterase were raised in New Zealand White rabbbits at the I.C.R.F. Clare Hall laboratories, Potters Bar, Herts, U.K. On day 1 of week 1, 10 ml of

preimmune serum was obtained before injecting, intra/subcutaneously, the rabbit with 200 μg of purified protein in Freund's complete adjuvent. At weeks 4 and 6 the rabbit was boosted with a further 100 μg of pure protein in Freund's incomplete adjuvent. 10 ml of test serum was obtained at week 7. The rabbit was given a final boost with 100 μg of protein in Freund's incomplete adjuvant at week 8. Rabbits were bled 9 days after the last boost and the serum isolated.

### 2.3.7 Human Serum Samples.

Sera were kindly provided by Dr. J. Gerry Kenna, St. Mary's Hospital Medical School. Sera from 20 patients with halothane hepatitis, defined clinically as otherwise unexplained hepatitis within 28 days of halothane anaesthesia were studied. They were aged 32-60 (median 53) and 17 were female. All had had halothane anaesthesia before, on between 1 and 8 occasions (median 2), and the interval between the current and previous or latest exposure ranged from 7 days to 10 years (median 6 weeks). 13 patients had liver failure and 12 died. Control sera were obtained from 9 patients who had recieved halothane several times without liver damage; from 18 healthy blood donors; and from 11 patients with fulminant hepatic failure (6 paracetamol, 2 hepatitis B, 1 hepatitis A, 2 non-A, and non-B hepatitis), 6 with autoimmune chronic active hepatitis, 4 with alcoholic liver disease, 8 with primary biliary cirrhosis (PBC) and 4 with jaundice due to flucloxacillin.

### 2.3.8 Immunohistochemical analysis of human and rat liver.

Tissue for immunohistochemical analysis was cut into small fragments and placed in formal saline (14% (v/v) formaldehyde in PBS)

PBS/litre: 1.5g $Na_2HPO_4$, 8 g NaCl, 0.1g $MgCl_2.6H_2O$, 1.5 g $KH_2PO4$, 0.2 g KCl, 0.132g $CaCl_2.2H_2O$

The tissue was paraffin embedded and sectioned at 3 μm. Sections were dewaxed in xylene and rehydrated through graded alcohols. Slides were washed several times in 100 mM Tris.HCl, pH 7.4, 0.1% Tween 20, 5% (v/v) normal goat serum, before overnight incubation with rabbit polyclonal human carboxylesterase antisera (diluted 1: 5,000 in the above buffer). The

slides were then washed, 3X 10 minutes, and incubated for 30 minutes with biotinylated goat anti-rabbit IgG (diluted 1:500 in the above buffer). Sections were exposed to streptavidin-peroxidase conjugate for 30 minutes before being developed with 3,3' diaminobenzidine and lightly counterstained with heamatoxylin.. Carboxylesterase localisation was then achieved using false colour image analysis via a Kontron system. Maximum intensity of antibody binding was signified by a blue colouration through green/yellow to red, signifying weak binding.

### 2.3.9 Isolation of microsomal fractions.

Tissue was added to 3 volumes of KCl-phosphate buffer (10 mM potassium phosphate, pH 7.4, 1.15% (w/v) KCl, 0.1 mM EDTA) and homogenised in a Silverson Laboratory mixer homogeniser at 4°C. The homogenate was centrifuged at 11,000g for 20 minutes to remove cell debris and larger cellular organelles such as mitochondria, nuclei and peroxisomes. The pellet was discarded and the supernatent centrifuged at 230,000g for 80 minutes, 4°C, to separate the microsomal and cytosolic fractions. The microsomal pellet was washed by resuspension in KCl-phosphate buffer using a Teflon-glass hand homogeniser and recentrifuged at 230,000g for 60 minutes at 4°C. The resulting pellet was resuspended by hand homogenisation in 0.25 M sucrose, 10 mM potassium phosphate buffer, pH7.7. Samples were stored at -40°C at a protein concentration of 15-30 mg/ml.

### 2.3.10 Enzyme Linked Immunosorbent Assay.

Purified carboxylesterase protein was prepared at a concentration of 2 µg/ml in 10 mM sodium phosphate buffer, pH 7.0. 50 µl of the protein was added to the wells of a 96-well polyvinylchloride microtitre plate and incubated at 4°C overnight. After removing the contents of the well, the plate was washed twice with PBS before filling the wells with 3% BSA in PBS for 2 hours at room temperature. The plate was then washed 2X with PBS before adding 50 µl of the appropriate sera at a dilution of 1:100. After incubation at room temperature for 1 hour the plate was washed twice with PBS before the addition of 50 µl of horse-radish peroxidase conjugated goat anti-human IgG at a dilution of 1:1000 in 3% BSA in PBS. After incubation at room temperature for 1 hour the plate was washed 3X with PBS. Substrate solution

was prepared as follows; 0.1 mg of o-phenylenediamine dihydrochloride (OPD) was dissolved in 0.1 ml of DMSO before making up to 10 ml with 0.1 M sodium acetate (pH 6.0). Hydrogen peroxide was then added to a final concentration of 0.01%. 50 µl of the substrate solution was added to each well and incubated for 20 minutes at room temperature. 50 µl of 1 M sulphuric acid was added to stop the reaction and the optical density at 492 nm measured.

### 2.4 Protein Purification Procedures

The major protein purification strategy employed in this study took into consideration the fact that along with P450 reductase it was hoped that isozymes of P450 and/or other hepatic drug metabolising enzymes would be purified. One of the major reasons for this was the value of human microsomes. This led to the development of a P450 reductase purification that was essentially similar to the one described by Yasukochi and Masters (1976) and a P450 purification that was similar to that described by Wolf *et al.* (1980). The purification was based around an initial step of ion-exchange chromatography on DEAE-cellulose so as to produce a pool of (i) partially purified P450 reductase and (ii) a pool of P450 isozymes for further purification. P450 reductase could then be purified by affinity chromatography on 2'5' ADP-sepharose. The pool of P450 isozymes would then undergo further chromatography on hydroxylapatite. However, on further purification of the P450 pool, two proteins were obtained in an electrophoretically homogenous form. At this stage and from the subsequent identification of one of these as a carboxylesterase it was decided that this protein should be the focus for further study (Chapter 5).
The solubilisation stage and all chromatography were performed at 4°C.

#### 2.4.1 Solubilisation of hepatic microsomes.

Microsomes were solubilised at a concentration of 10 mg/ml in 10 mM potassium phosphate buffer, pH 7.7 containing 0.1 mM dithiothreitol and 0.1 mM EDTA, by the slow addition of a 10% (w/v) solution of sodium cholate to give a final concentration of 1 mg detergent per 1 mg microsomal protein. After 30 minutes of stirring, glycerol was added to give a final concentration

of 20% v/v. The solubilised microsomes were then subjected to centrifugation (100,000 g, 30 minutes) to remove any unsolubilised material/ debris.

### 2.4.2 Ion exchange chromatography using DEAE-cellulose.

DEAE-cellulose (Whatman DE52) was prepared according to the manufacturers instructions. A column of DEAE-cellulose was prepared (5 x 90 cm) and equilibrated with 10 mM buffer A (potassium phosphate buffer, pH 7.7 containing 20% glycerol, 0.1 mM dithiothreitol and 0.1 mM EDTA). Once the column was equilibrated, the solubilised microsomes were applied at a flow rate of 120ml/ hour. The column was washed extensively with 10 mM buffer A + 0.2% sodium cholate. By increasing the ionic strength of buffer A to 30 mM and by the addition of Emulgen 911 to 0.1% (v/v) a pool was eluted that was rich in cytochrome P450 and carboxylesterase. By applying a 0-500 mM KCl gradient to the column a pool of P450 reductase was eluted at around 250 mM. The pool of P450 reductase was identified by cytochrome c reductase activity (2.5.2).

### 2.4.3 2'5' ADP-sepharose chromatography.

The affinity resin 2'5' ADP-sepharose interacts strongly with $NADP^+$ dependent dehydrogenases and other enzymes which have affinity for $NADP^+$. The reductase can be sucessfully eluted from the column by the use of 2' AMP which has a slightly lower binding constant for the reductase than $NADP^+$.

The P450 reducatse pool obtained after DEAE-cellulose chromatography was applied to a 2' 5' ADP- Sepharose column (10 x 1.5 cm) pre-equilibrated with 10mM buffer A + 0.2% v/v sodium cholate at a flow rate of 25 ml/ hour. The bound P450 reductase was washed extensively with the equilibration buffer and eluted by the addition of 2'AMP to a final concentration of 0.7 mM. Any remaining P450 reductase was eluted by the addition of 0.1% (v/v) Emulgen 911 to the buffer. The pooled P450 reductase was dialysed extensively against 10mM buffer A and stored in aliquots of approximately 1 mg/ml at -40°C after concentration using an Amicon concentrator with a PM30 membrane. Purity of fractions was assessed by SDS-PAGE (2.3.2).

A modified version of this procedure was used when purifying expressed rat P450 reductase from *S. typhimurium* (Chapter 3). 10 mls of an overnight

culture of TA1535, harbouring the rat P450 reductase expression vector pMP230, was added to 1 litre of fresh L.B. broth containing ampicillin, 50 μg/ml. After 1.5 hours, isopropyl-1-thio-β-D-thiogalactopyrannoside was added to a final concentration of 1 mM. After 4 hours expression the cells were harvested, 5000 g for 10 minutes, and resuspended in 10 mM potassium phosphate buffer, pH 7.7, 20% glycerol and 0.1 mM DTT (3 ml buffer/ 1 g cells). Lysozyme was added to a final concentration of 1 mg/ml and left at 4°C for 30 minutes with rotary mixing followed by the addition of Triton X-100 to a final concentration of 0.1% and mixed for a further 10 minutes. 0.1 volumes of 4% sodium deoxycholate was subsequently added and the resulting lysed bacteria centrifuged at 230,000g for 1 hour. The supernatent was removed, applied to 2' 5' ADP-sepharose and chromatography carried out as described above. The purified recombinant rat P450 reductase was purified in the one-electron reduced air-stable semi-quinone form, as judged by spectral analysis.

### 2.4.4 Hydroxylapatite chromatography.

In order to resolve the carboxylesterase and P450 containing pool obtained from the DEAE-cellulose step, hydroxylapatite chromatography was employed.

A 2.5 x 20 cm hydroxylapatite (Biorad HT) column was prepared and equilibrated with 10 mM buffer A. The carboxylesterase/ P450 containing pool was diluted 5 fold with 10 mM buffer A and applied to the column at a flow rate of 40 ml/ hour. After extensive washing with 10 mM buffer A, elution of proteins took place in a stepwise manner by increasing the phosphate concentration of buffer A in 10 mM steps in the presence of 0.1% (v/v) Emulgen 911. In this way a variety of microsomal proteins were resolved as judged by SDS-PAGE analysis. It is important to remember that Emulgen 911 has a very high absorbance at 276nm and hence protein elution cannot be carried out by monitoring at 280nm.

### 2.4.5 Concanavalin A-Sepharose chromatography.

Concanavalin A (con A) binds molecules which contain α-D-mannopyranosyl, α-D-glucopyranosyl and sterically related residues. The binding sugar requires the C-3, C-4 and C-5 hydroxyl groups for reaction with con A (Goldstein *et al.*, 1965).
Since microsomal carboxylesterases have been shown to be glycosylated (Harano *et al.*, 1988) it was felt that by using con A-sepharose chromatography any remaining protein impurities along with Emulgen 911 would be removed. A con-A sepharose (Pharmacia) column (0.5x 5 cm) was equilibrated with 10 mM buffer A and to it was applied hydroxylapatite purified carboxylesterase at a flow rate of 20 ml/ hour. After extensive washing of the bound carboxylesterase with 10 mM buffer A, carboxylesterase was eluted from the resin by the addition of 0.2 M α-methylmannoside. Samples were dialysed extensivley against 10 mM buffer A and stored at -40°C.

### 2.4.6 Deglycosylation and glycosylation studies.

Deglycosylation was carried out by incubating 10 μg of purified carboxylesterase with 2 units of N-glycosidase F in 50 mM potassium phosphate buffer, pH 7.7 containing 10 mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol at 37°C overnight. Loss of sugar residues was visualised by SDS-PAGE on a pre-cast 10-15% acrylamide gradient gel on a Pharmacia Phast System.
Glycans were identified after transferring purified carboxylesterase resolved on a 10-15% acrylamide gel onto PVDF membranes, using a glycan differentiation kit (Boehringer Mannheim) according to the manufacturers instructions.

### 2.4.7 Purification of the domains of P450 reductase.

Cloning into the *Nde*I/*Xho*I sites of pET15b engineers a 6x His-linker and a thrombin cleavage site onto the $NH_2$-terminus of the expressed protein. Protein purification of the His-tagged domains was carried out using nickel-agarose chromatography. BL21 strains harbouring the domain expression plasmids were grown at 37°C overnight in L.B. broth containing 50 μg/ml

ampicillin and 34 μg/ml chloramphenicol. 500 ml of fresh LB broth was inoculated with 5 ml of the overnight culture and grown at 37°C to an O.D. of 0.5-0.6. Isopropyl-1-thio-β-D-galactopyrannoside was then added to give a final concentration of 0.5 mM and the culture allowed to grow for a further 2.5 hr before harvesting at 5000 g for 10 min. The bacterial pellet from a 500 ml culture was resuspended in 20 ml of 'binding buffer' (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) and frozen at -70°C for 1 hr. Upon thawing the bacteria were lysed, since the expression strain BL21 contains a plasmid bearing T7 lysozyme. Sonication, using an MSE soniprobe at full power (2x 35s with 1 min incubation on ice between steps), was carried out to shear genomic DNA present in the lysed bacteria. The bacterial extract was then centrifuged (39,000g for 30 min) to remove cellular debris and insoluble protein. Bacteria were analysed for domain expression by SDS-PAGE of soluble and insoluble fractions. The FMN/anchor, FMN, FMN (small) and FAD/NADPH domains produced soluble protein extracts. However, the FAD, FAD/NADPH(small) domains were insoluble, as was the FMN/FAD domain with only a very small percentage present in the soluble fraction. The supernatant containing the soluble domains was applied onto 2 ml nickel-agarose columns previously equilibrated with 'binding buffer'. Columns were washed with 10 volumes of 'binding buffer' and then 10 volumes of 'wash buffer' ('binding buffer' containing 20 mM imidazole). The FMN(small) domain was found to elute in the 'wash buffer'. The FMN (no anchor) and the FAD/NADPH domains could be eluted from the nickel-agarose column by increasing the imidazole concentration to 60 mM, while the FMN/anchor region was eluted by increasing the imidazole concentration to 300mM. Insoluble proteins were purified in a manner similar to the soluble domains but in the presence of 6 M urea. Attempts to renature the insoluble domains were unsuccessful. The purity of the domains was determined by SDS-PAGE. The insoluble domains were not used for any further experiments. Purified soluble domains were extensively dialysed against 10 mM potassium phosphate buffer, pH 7.7, 20% glycerol and stored at -40°C

### 2.4.8. Removal of the 6x- His -Tag by thrombin cleavage.

Thrombin cleavage was performed in 20mM Tris-HCl, pH 8.4, 150mM NaCl and 2.5 mM $CaCl_2$ with a w/w domain to thrombin ratio of 5000:1. Cleavage

took place at 4°C and was monitored at various times by SDS-PAGE. 100% cleavage occurred after 10 min for the FMN (no anchor) domain and after 4 hr for the FAD/NADPH domain. The His-tag could not be removed from the FMN/anchor domain. PMSF was added to a final concentration of 0.1 mM to inhibit the thrombin after removal of the His-tag. The cut domains were separated from the His-tag by passing the sample through a nickel-agarose column. Cleaved proteins were dialysed against 10 mM potassium phosphate buffer, pH 7.7, 20 % glycerol and stored at -40°C

### 2.5. Biochemical Assays.

#### 2.5.1. Spectral Analysis.

Absorption spectra were obtained using a Shimadzu U.V. 2000 spectrophotometer. FMN and FAD content was calculated after releasing the flavin from the domains by boiling for 3 min in the dark, followed by centrifugation (39,000g for 10 min) to remove denatured protein. The flavin concentration was determined at 450nm using extinction coefficients of 12.2 $mM^{-1}cm^{-1}$ for FMN (Whitby, 1953) and 11.3 $mM^{-1}cm^{-1}$ for FAD (Beinhert, 1960).

#### 2.5.2. P450 reductase assays.

A variety of assays were perfromed to study the one-electron reduction ability of bacterially expressed P450 reductase and the domains of P450 reductase. These assays were all taken from the work of Vermillion and Coon, (1978a) and carried out at 37°C in 50 mM potassium phosphate buffer, pH 7.7 using a Shimadzu U.V. 2000 spectrophotometer. Apart from the reconstitution of the domains of P450 reductase, cytochrome c reduction was carried out in 300 mM potassium phosphate buffer , pH 7.7. Compounds were obtained from Sigma. The reduction of horse heart cytochrome c (40 μM) was followed at 550 nm using an extinction coefficient of 21,400 $M^{-1}$ $cm^{-1}$. The reduction of dichlorophenolindophenol (50 μM) was followed at 600 nm using an extinction coefficient of 21,000 $M^{-1}$ $cm^{-1}$. The reduction of potassium ferricyanide (500 μM) was followed at 420 nm using an extinction coefficient of 21,400 $M^{-1}$ $cm^{-1}$. The reduction of menadione (50 μM) was followed indirectly by measuring the reduction of NADPH using an extinction

coefficient of 6,220 $M^{-1}$ $cm^{-1}$ at 340 nm. The reduction of 3-acetyl pyridine adenine dinucleotide phosphate (500 μM) was followed at 363 nm using an extinction coefficient of 5,600 $M^{-1}$ $cm^{-1}$.

**2.5.3. Reconstitution of cytochrome P450 monooygenase activity.**

Reconstitution of cytochrome P450 monooygenase activity was carried out using purified rat P450 (CYP1A1) and purified rat P450 reductase or a mixture of the FMN/anchor and FAD/NADPH domains. CYP1A1 was obtained previously by the method of Wolf and Oesch (1983) and rat P450 reductase obtained by the method described above (Yasukochi and Masters, 1976). The O-deethylation of 7-ethoxyresorufin was followed by measuring the rate of resorufin formation (Burke and Mayer, 1974) An excitation wavelength of 535nm and an emission wavelength of 585nm were used. The concentration of substrate was 1 μM and the assays performed at 37°C in 10 mM potassium phosphate buffer, pH 7.7. For reconstitution using the FMN/anchor and FAD/NADPH regions of human P450 reductase, domains (0.1 nmol) were mixed in 10 mM potassium phosphate buffer, pH 7.7 in a volume of 20 μl at 4°C. After various times or in varying concentrations of buffer, the domains were incubated with dilaurylphosphatidylcholine (25 μg) and CYP1A1 (37.5 pmol in 5 μl) at 37°C for 5 minutes before measuring the rate of resorufin formation (Burke and Mayer, 1974).

**2.5.4 Effect of human P450 reductase domains on monooxygenase activty.**

CYP1A1 (7.5 pmol) and 25 μg of dilaurylphospatidylcholine were incubated for 5 minutes with varying concentrations of the FMN/anchor, FMN or FAD/NADPH domains (0.04-1.7 nmol) in a total incubation volume of 47.5 μl at 37°C for 5 minutes. Native P450 reductase (0.05 nmol in 2.5 μl) was then added and the sample incubated for a further 5 minutes before measuring 7-ethoxyresorufin O-deethylation. Free FMN was also used in experiments at the same concentrations to the domains used.

### 2.5.5. Carboxylesterase Assay.

Esterase activity was determined by measuring the formation of p-nitrophenol using p-nitrophenyl acetate (1.6 mM) at 405nm (Krisch, 1966) Incubations were at 37°C in 50 mM Tris.HCl buffer, pH 8.0. Inhibition studies were carried out by incubating carboxylesterase in varying concentrations of phenylmethylsulphonyl fluoride and bis-4-nitrophenyl phosphate for 30 minutes at 37°C prior to assay.

### 2.5.6. Superoxide dismutase Assay.

Superoxide dismutase (SOD) assays were performed on soluble extracts from *E. coli.* (39,000g,supernatant) after 2x 30 second sonication bursts using an M.S.E. soniprobe. The SOD assay was performed according to the method of McCord and Fridovich, 1969. The assay was carried out in 50 mM Tris.HCl, pH 7.4 containing 1 mM $MgCl_2$. The reaction mixture contained 10 μM cytochrome c, 50 μM xanthine and xanthine oxidase to a concentration (around 5 nM) that produced a rate of reduction of cytochrome c at 550nm of 0.025 A.U. per minute. Under these conditions the amount of superoxide dismuthase required to inhibit the rate of reduction of cytochrome c by 50% (i.e. to a rate of 0.0125 A.U. per minute) is defined as one unit of activity.

### 2.5.7. Catalase Assay.

Catalase assays were performed on soluble extracts from *E. coli.* (39,000g, supernatant) after 2x 30 second sonication bursts using an M.S.E. soniprobe. The catalase assay was performed according to the method of Beers and Sizer (1952). The assay was performed in 50 mM potassium phosphate buffer, pH 7.0 containing 5 mM hydrogen peroxide. Destruction of hydrogen peroxide was followed at 240 nm.

# Chapter 3: Expression of rat NADPH cytochrome P450 oxidoreductase in *S. typhimurium* and *E. coli* for the study of one-electron reduction reactions.

## 3.1 Introduction and aims.

One method to assess the role of individual drug metabolising enzymes in the metabolism of xenobiotics is by the use of heterologous expression systems (Simula *et al.*, 1993a). The use of yeast and bacterial systems have been exploited a great deal for this purpose (Bloch *et al.*, 1986; Watanabe *et al.*, 1987; Black *et al.*, 1989; Simula *et al.*, 1993b; Simula *et al.*, 1993c). The attraction of these systems stems from the fact that, unlike mammalian cells, there is a low background of endogenous xenobiotic metabolising enzymes, making results for a particular enzyme more easily interpretable. Cytotoxic effects on bacteria or yeast can simply be assessed by plating chemically exposed cells onto nutrient agar and comparing the survival frequency of enzyme expressing and non-expressing cells. A major reason for the use of heterologous expression systems for studying individual enzymes has been the advent of recombinant DNA technology. Heterologous expression systems are not only of value for cytotoxicity or mutagenicity studies but for the study of enzymes which are present in low abundance where their isolation maybe very difficult (Miles and Wolf, 1991).

Advantages of yeast and bacterial systems over mammalian cells for heterologous expression is their ease of genetic manipulation, where controlled and stable expression of foreign proteins is more easily achievable. The simplistic nature of yeast and bacteria has enabled detailed study of their transcription and translation machinery to be made. This has subsequently led to the construction of efficient expression vectors with promoters which are able to express the foreign protein of interest. However, successful expression of mammalian proteins is not always achieved using yeast and bacterial systems. Many explanations abound for the failure of these systems to express foreign proteins. For example, limitations in translation maybe encountered since there is a marked difference in codon usage between organisms. Differences can exist in RNA processing, post-translational modifications and the misreading of cellular targeting signals. The size of proteins and their hydrophobicity can also present problems as well as unwanted proteolysis (Goeddel, 1990).

One criticism of using heterologous expression systems to study the functional properties of an expressed enzyme is that foreign proteins in the cell system may have adverse effects on the host cells normal functions. Therefore, this may alter the cells sensitivity to a chemical toxicity. An example of this has been in the study of superoxide dismuthase (SOD) overexpression in *E.coli* (Bloch *et al.*, 1986). Rather than protect against the cytostatic effects of the superoxide generating compound paraquat it was found that SOD expressing bacteria were more sensitive to the cytostatic effects of paraquat than non-expressing bacteria. This paradox was explained by Liochev and Fridovich (1991) who showed that SOD expression interfered with the induction of the protective *sox RS* system. This reduced the ability of the SOD expressing bacteria to adapt to the presence of paraquat compared to non-expressing bacteria.

Earlier studies from this laboratory have shown that P450 reductase has the ability to enhance the cytotoxic properties of the redox cycling anticancer agents mitomycin C and doxorubicin (Bligh *et al.*, 1991 and Bartoszek and Wolf, 1992). These studies were performed by exogenously treating the cells with the drug, P450 reductase and NADPH. The study on the capacity of P450 reductase to bioactivate mitomycin C also used an *S.typhimurium* strain (LR5000) expressing rat P450 reductase. In cytotoxicity assays it was shown that the P450 reductase expressing strain was more sensitive to mitomycin C than the non-expressing strain. This implicated P450 reductase in the bioactivation of mitomycin C *in vivo*. Other work has looked at the overexpression of GSTs in Ames Test strains to increase or decrease the mutagenicity of a variety of compounds (Simula *et al.*, 1993b, Simula *et al.*, 1993c). Therefore, previous work has shown the usefulness of heterologous expression sytems for the study of drug metabolising enzymes.

The aims of this chapter were to study the effects of P450 reductase overexpression in *S. typhimurium* on the cytotoxic actions of the redox cycling compounds paraquat, menadione and the anticancer agent doxorubicin. Low levels of P450 reductase expression were obtained in the previous work of Bligh *et al.* (1991) when using the *S. typhimurium* strain LR5000. To overcome this the use of two, more efficient, bacterial expression systems were decided upon. This chapter will discuss the expression of rat P450 reductase in *S.typhimurium* and *E.coli* using the modified pKK233-2 (pGLW11) and pOR263 expression vectors. The effects of the expression of P450 reductase on the cytotoxicity to *S. typhimurium* by the aforementioned

compounds will be discussed. While this work was being carried out extensive analysis of the *sox RS* and *oxyR* regulons in *E.coli* became available (Demple and Amabile-Cuevas, 1991; Storz *et al.*, 1990). The ability of P450 reductase expression in *E.coli* to modulate these regulons when exposed to the redox cycling compounds was also investigated. These latter experiments were carried out in *E.coli* since these regulons have been more fully characterised in this bacteria.

### 3.2-3.4 Results and discussion.

### 3.2.1 Heterologous expression of rat NADPH cytochrome P450 oxidoreductase in *E.coli* and *S.typhimurium*.

To express a eukaryotic protein in a bacterial system modifications have to be made to the eukaryotic transcript before it will be translated in the prokaryote. Bacterial transcripts contain a conserved sequence just 5' of the initiation methionine codon termed the Shine-Dalgarno (S.D.) or ribsomal binding sequence (RBS) (Shine and Dalgarno, 1974). This sequence shows a variable degree of complementarity to a region of the ribosomal 16S RNA (Atkins, 1979) In general the RBS has four nucleotides from the sequence AGGAGG placed at a distance of between 5 and 9 base pairs from the initiation codon, ATG. Therefore, eukaryotic messages need to be modified by the addition of an appropriately spaced S.D. sequence upstream of the initiating codon. Other modifications that have been included to enhance the expression of a mammalian protein in a prokaryote include altering the codon bias to that of the prokaryote and replacing structural sequence after the initiation codon with unstructured sequence (Barnes *et al.*, 1991). The expression vectors used in this study were a modified version of pKK223-3 (Pharmacia) termed pGLW11 and pOR263 (Shen *et al.*, 1989). The former vector contains a promoter derived from the fusion of the bacterial *trp* gene promoter, a partial Pribnow box, a Shine-Dalgarno sequence and a *lac*-operator sequence from the *lac UV5* gene. Collectively this is called the *tac II* promoter. If the host strain harbouring the plasmid produces the repressor protein encoded by the *lac I* gene the promoter is transcriptionally inactive. However, addition of isopropyl-1-thio-β-D-thiogalactopyrannoside (IPTG) leads to the dissociation of the bound repressor from the operator sequence allowing transcription to procede. pGLW11 also contains a copy of the *lac Iq*

gene which results in the over-production of the *lac I* repressor. This basically means that pGLW11 does not require a *lac Iq* host strain. The modification of the vector pKK223-2 was carried out by Dr I. Hunter, University of Glasgow. The expression plasmid pOR263 utilises the *trp* promoter and encodes the first 12 amino-acids of the outer membrane protein A (ompA) signal peptide. By cloning in frame with the ompA leader sequence the resulting expressed protein is exported to the periplasm of the bacteria. This expression vector containing the rat P450 reductase cDNA was generously provided by Dr. Charles. B. Kasper, University of Wisconsin (Shen *et al.* 1989). Construction of the P450 reductase expression plasmid, pMP230, was performed by cloning the rat cDNA into the expression vector pGLW11 (described above). This cloning was performed in this laboratory in collaboration with Pauline van der Drift. A *Bam* HI fragment containing the rat P450 reductase cDNA was isolated from the plasmid pJLA1 (Bligh *et al.*, 1991), blunt ended using nuclease S1 and ligated into the Klenow filled *Eco* RI site of pGLW11. Clones were obtained with the cDNA in the correct and incorrect orientation, the latter being used for control experiments. Figure 3.1 shows SDS-PAGE analysis of both insoluble (39,000g pellet) and soluble extracts (39,000g supernatent) from *E.coli* (NM522) and *S. typhimurium* (TA1535) expressing and non-expressing bacteria. What can clearly be seen from these gels is that the majority of the expressed protein after both 4 and 22 hours is found in the insoluble fraction of the bacteria. Higher levels of expressed rat P450 reductase can be seen to be produced from the pOR263 expression plasmid. Interestingly, higher levels of expression can be seen in the *S. typhimurium* strain than in the *E. coli*. Of particular note from this gel is that the protein produced by the pMP230 expression vector is smaller than that expected for P450 reductase (compare 65 kDa. with 78 kDa.).

Analysis of cytochrome c reductase activity found in both the soluble and insoluble extracts of *E.coli* (NM522) and *S.typhimurium* (TA1535) is shown in Figure 3.2. Samples were analysed at both 4 and 22 hours. Similar to the levels of expressed protein observed by SDS-PAGE, the *S. typhimurium* strain and the pOR263 derived P450 reductase had the greater capacity to reduce cytochrome c. Interestingly, Simula *et al.* (1993a) found that the heterologous expression of human GST isozymes was elevated in *S.typhimurium* by at least 2-3 fold over that found in *E. coli* strains. Expression of a mouse cytochrome P450 (Cyp2E1) was also found to be elevated when expressed in *S. typhimurium* compared to *E. coli* (Freeman, 1993). These previous

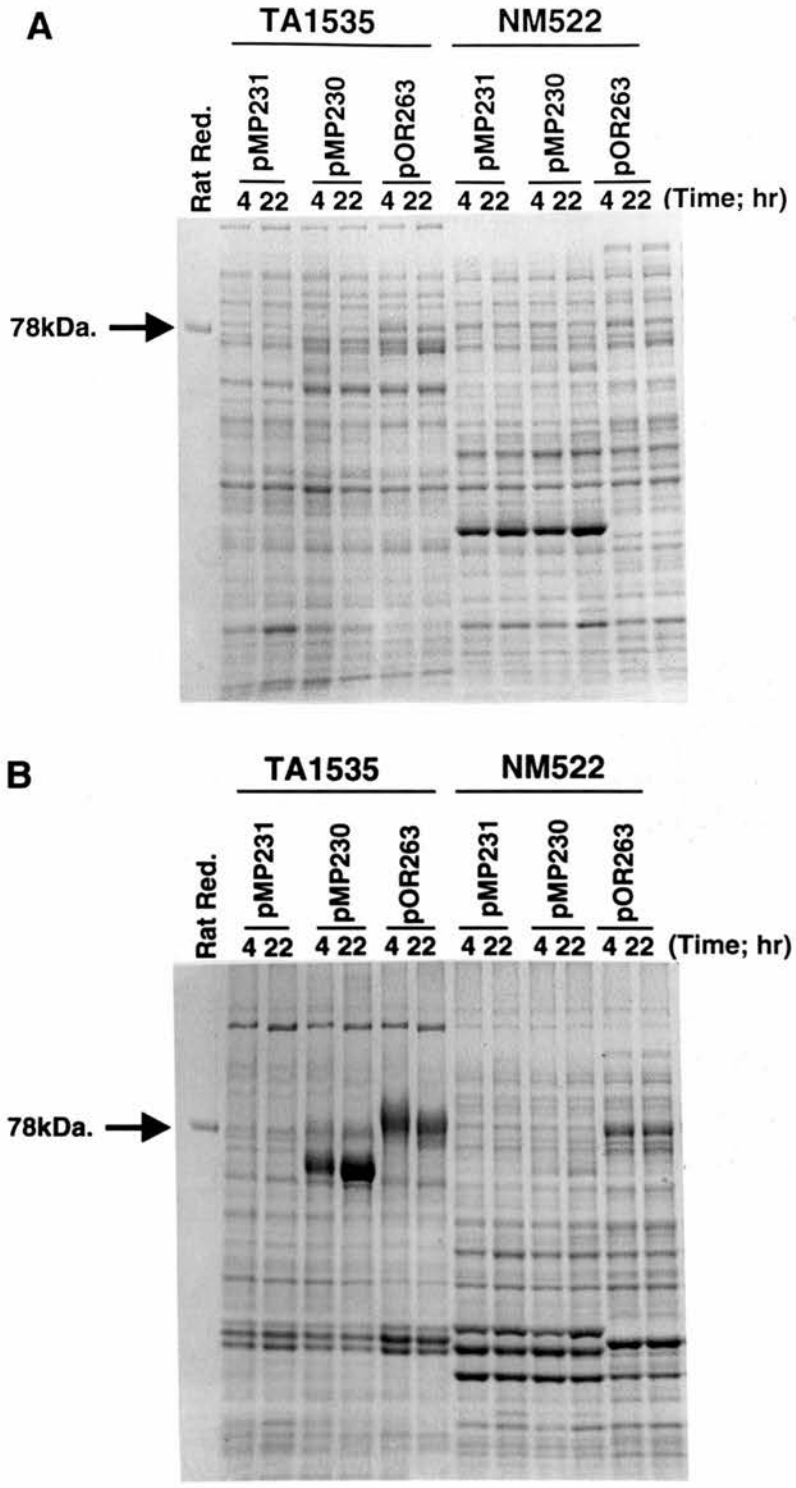



**Figure 3.1. SDS-PAGE (9%) analysis of expressed rat P450 reductase in *E.coli* (NM522) and *S.typhimurium* (TA1535).** (A) soluble fraction (39,000g supernatent). (B) insoluble fraction (39,000g pellet). Expression vectors used are shown, as are times of harvest after IPTG induction. Rat red, purified native rat P450 reductase (2 μg).

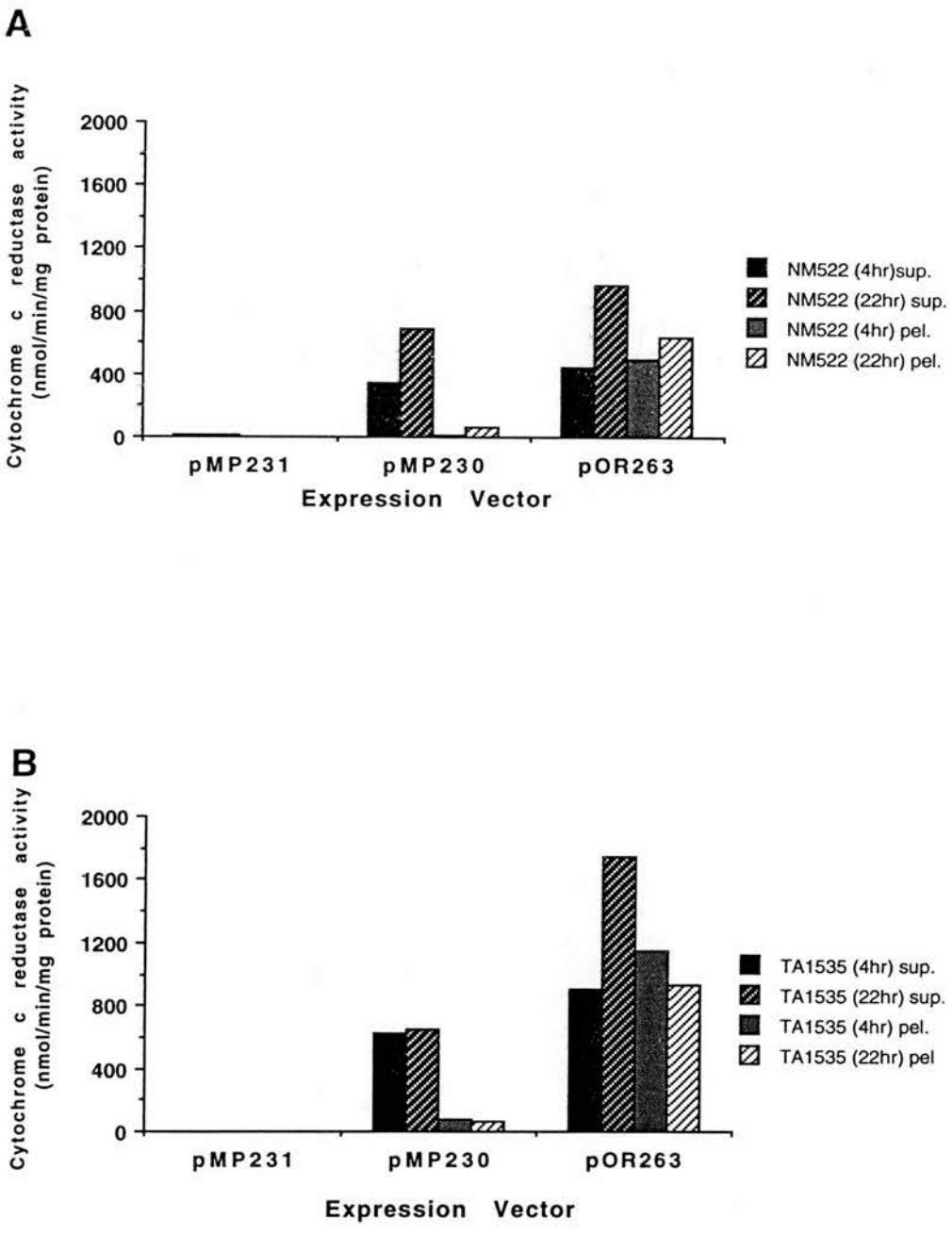



**Figure 3.2. Cytochrome c reductase activity of expressed rat P450 reductase in (A) *E. coli* (NM522) and (B) *S. typhimurium* (TA1535).** Assays were performed after 4 and 22 hours of IPTG induction. Results are the mean of two seperate experiments performed in triplicate. Sup, 39,000g supernatent; pel, 39,000g pellet.

observations for the GST isozymes and Cyp2E1 do indeed appear true for the rat P450 reductase expressed here. The mechanisms for this higher level of expression in *S. typhimurium* over *E. coli* are not clear and maybe due to either a more efficient initiation of transcription and/or translation or a reduced level of protein turnover.

### 3.2.2. Purification and characterisation of the 65 kDa. form of recombinant rat P450 reductase.

Expression of the rat P450 reductase cDNA in both *E.coli* and *S.typhimurium* utilising the expression vector pGLW11 resulted in the production of a 65 kDa protein which appeared highly active in the one-electron reduction of cytochrome c. In order to further characterise this protein it was purified from the soluble fraction of *S.typhimurium* using a one step affinity purification with the matrix 2'5' ADP-Sepharose (Figure 3.3). This one step procedure resulted in a 95% purified protein which was highly active in the reduction of cytochrome c, 48,000 nmol cytochrome c reduced / minute/ mg protein. Spectral analysis revealed the protein to contain both flavins and to have been purified from the bacteria in the one-electron reduced air-stable semiquinone form.

These results suggested that the protein was degraded from the $NH_2$-terminus. Sequence alignments of P450 reductase with spinach ferredoxin $NADP^+$ reductase (whose crystal structure is known) suggest that the last three residues of P450 reductase are critical for FAD binding (Karplus *et al.*, 1991) along with other residues in the C-terminal portion that are important for NADPH binding and therefore catalysis and interaction with the affinity resin. It can be postulated that degradation was occuring from the $NH_2$-terminus of the protein. To confirm this the purified 65 kDa. P450 reductase was transferred onto a PVDF membrane and the amino-acid sequence of the $NH_2$-terminus obtained by solid phase sequencing. The sequencing was kindly performed by Dr. Darryl Pappin, I.C.R.F., London. The sequence that was obtained revealed that the first 69 amino-acids of the protein were not present. This results in the loss of a predicted 7.8 kDa. of the protein. This region of P450 reductase encodes the membrane anchoring region and the proposed bridge between the anchor and the predicted FMN domain (Porter and Kasper, 1990). Therefore, it would appear that the bacteria are efficiently dissecting the rat P450 reductase into hydrophobic and hydrophilic portions.

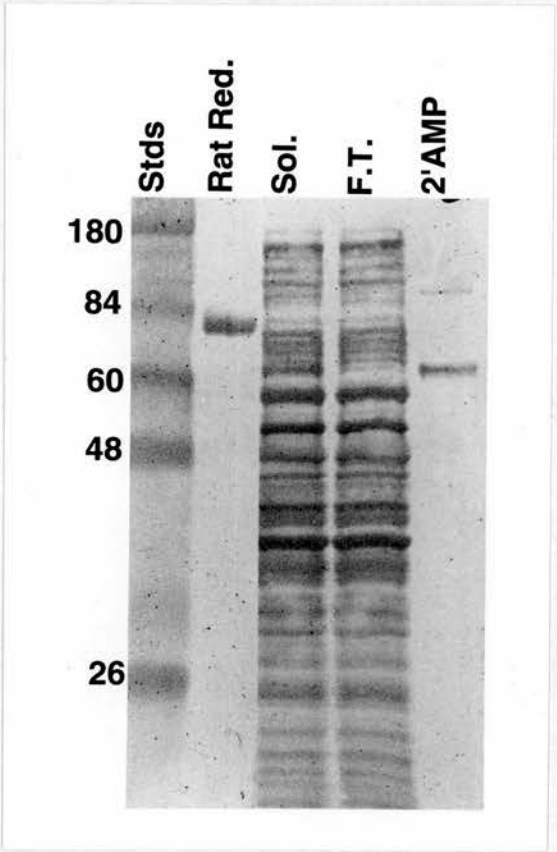



**Figure 3.3. SDS-PAGE analysis (9% gel) of the 2'5' ADP-sepharose affinity purification of the 65 kDa. form of rat P450 reductase expressed in *S. typhimurum* (TA1535).** Stds, prestained molecular weight standards ; rat red., native rat P450 reductase (2μg); sol., soluble fraction loaded onto 2'5' ADP-sepharose; F.T., flow through; 2'AMP, fraction eluted with 0.7 mM 2'AMP.

A peptide map of the protein, produced by the computer program 'Pepmap' from the GCG package reveals that a chymotrypsin site is present at this part of the rat P450 reductase protein. It can be suggested that a chymotrypsin like enzyme present in *E.coli* is causing this cleavage. The rat reductase expressed from the plasmid pOR263 was found not to be degraded. This is because at the $NH_2$-terminus of the protein there is the signal peptide sequence for the ompA protein of *E.coli*. This leader sequence will transport the protein to the periplasm where it will be protected from degradation (Shen *et al.*, 1989). Despite the degradation of the protein expressed from the pGLW11 vector, the 65 kDa form of rat P450 reductase was still highly efficient in cytochrome c reduction and appeared to bind both its flavins. A large scale purification of this degraded protein has enabled crystallisation trials to be set up in collaboration with Dr. Huub Driessen, Birkbeck College, London. Since hydrophobic proteins are inherently more difficult to crystallise, the removal of the anchor region of P450 reductase may prove of value in attempts at crystallisation.

## 3.3 Effects of redox cycling agents on the viability and growth of rat P450 reductase expressing and non-expressing *S. typhimurium* strains.

### 3.3.1. Overview of redox cycling.

A summary diagram of redox cycling showing the production of reactive oxygen species is shown in Figure 3.4. It is both the active oxygen products produced and the one-electron reduced compound that can result in cellular damage whether at the level of DNA, lipid or protein. In general, the compound is reduced in a one electron step to a reactive intermediate which is able to transfer one electron to molecular oxygen resulting in the formation of the superoxide anion radical. Only if the reductase involved is not inhibited by dioxygen can redox cycling occur. The enzymes involved in catalysing redox cycling of foreign compounds are flavoproteins with relatively low substrate selectivity (Kappus, 1986). Therefore, the number of enzyme systems being able to catalyse such a reduction step is limited. As shown in Figure 3.4, with quinonoid compounds, semiquinone radicals are formed which can go on to reduce dioxygen. (Paraquat, methyl viologen, a dipyridyl is also a very effective redox cycling compound.) The superoxide radical ($O_2^{\cdot-}$)

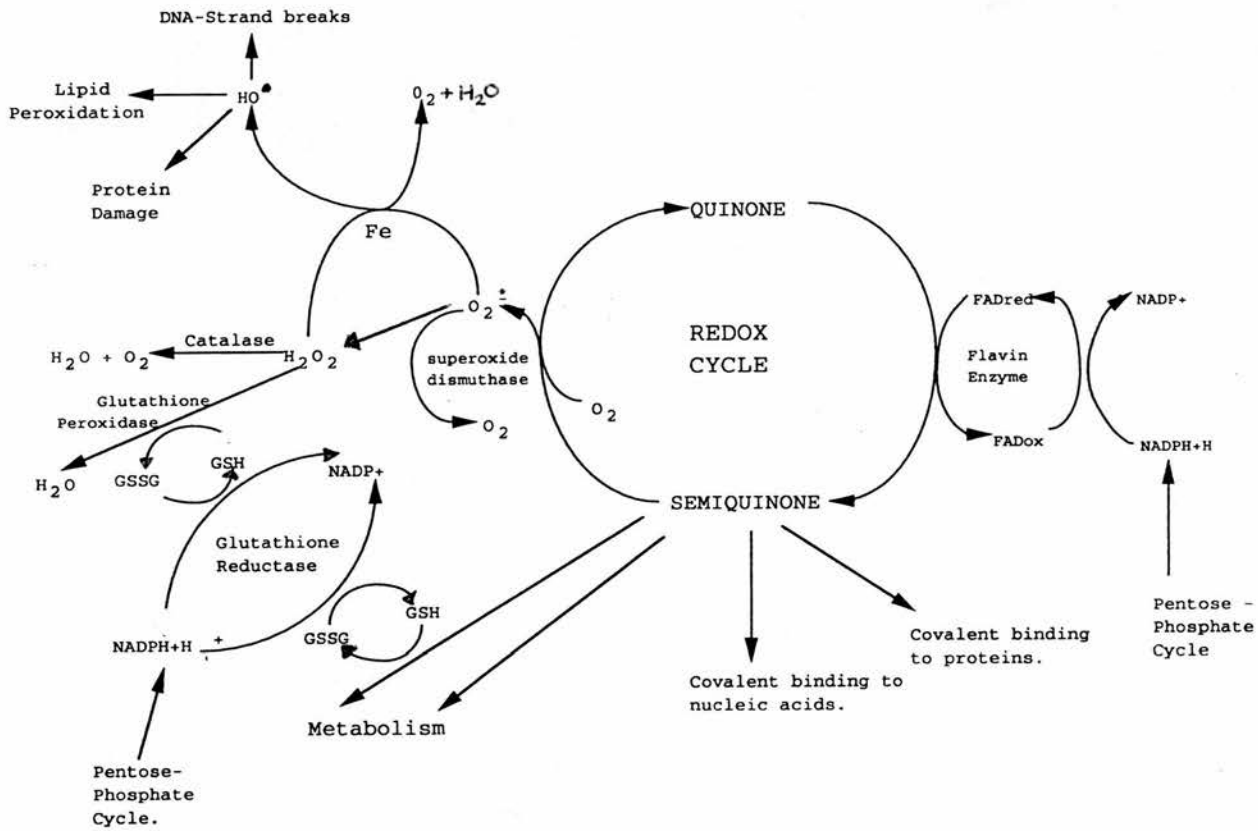
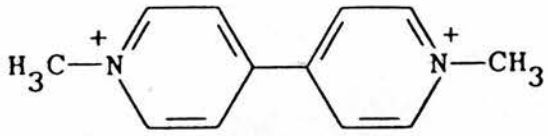



**Figure 3.4. Overview of redox cycling of quinone containing compounds.** (Modified from Kappus (1986)).

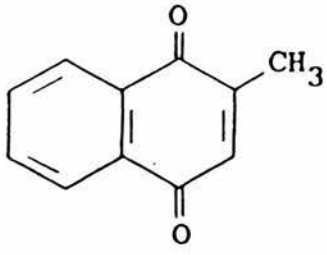
that is formed can then undergo a variety of reactions. $O_2^-$ will oxidise thiols, ascorbate, tocopherol and catecholamines . Proteins containing $FeS_4$ clusters are highly sensitive to attack by $O_2^-$ (Gardner and Fridovich, 1991). Probably the most important reaction of $O_2^-$ is its dismutation to hydrogen peroxide and its ability to reduce transition metals and metal complexes. The relevant transition metals *in vivo* are $Fe^{3+}$ and $Cu^{2+}$. The reduction of metal ions leads to the so-called metal catalysed Haber-Weiss cycle. The reduction of cupric and ferric ions by $O_2^-$ can occur even when the metals exist in a complexed form. For example, cytochrome c ($Fe^{3+}$) can be reduced to cytochrome c (Fe2+) by $O_2^-$ (McCord and Fridovich, 1969). Hydrogen peroxide produced can be removed either by catalase or by glutathione peroxidase. Hydrogen peroxide produced is most likely responsible for hydroxyl radical formation which occurs in the presence of reduced metals like ferrous ions. The hydroxyl radical is the most reactive oxygen metabolite and is suggested to be responsible for some of the serious damage occurring during redox cycling processes e.g. peroxidation of membrane lipids, protein, DNA damage (Kappus, 1986). Lipid peroxidation changes cellular integrity and releases toxic reaction products (Kappus, 1985). Protein damage by oxygen radicals leads to amino-acid oxidations resulting in conformational changes and enzyme inactivation (Mason, 1982). While DNA damage by hydroxyl radicals can lead to cytotoxicity, mutagenicity and carcinogenicity.
In an attempt to assess the role of the flavoprotein NADPH cytochrome P450 oxidoreductase in reducing a quinone (menadione), an anthracycline (doxorubicin) and the dipyridal compound paraquat *in vivo* the *S. typhimurium* strains described above (3.2.1, 3.2.2) were used.

### 3.3.2 Cytotoxic and growth inhibitory effects of paraquat on P450 reductase expressing and non-expressing *S. typhimurium*.

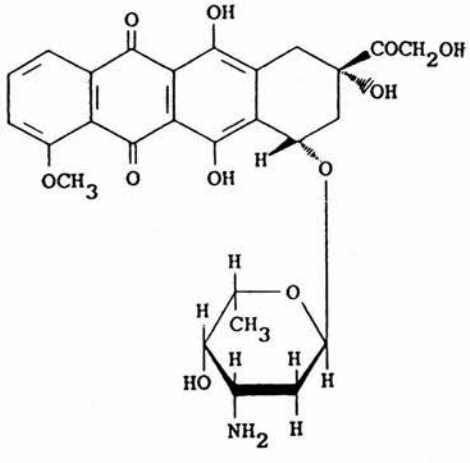
Paraquat (1,1-dimethyl-4,4'-bipyridilium; methyl viologen) is a non-selective contact herbicide (Figure 3.5). The paraquat dication is known to undergo cyclic oxidation-reduction (Bus *et al.*, 1974). Enzymatic one-electron reduction of the paraquat dication results in formation of the paraquat cation free radical. In the presence of oxygen the paraquat cation radical is immediately reoxidised and the superoxide radical immediately generated (see Figure 3.4). The cytotoxic effects of varying concentrations of paraquat on the *S.typhimurium* strains expressing and non-expressing P450 reductase are



**Paraquat**



**Menadione**



**Doxorubicin**

**Figure 3.5**. Chemical structures of paraquat, menadione and doxorubicin.

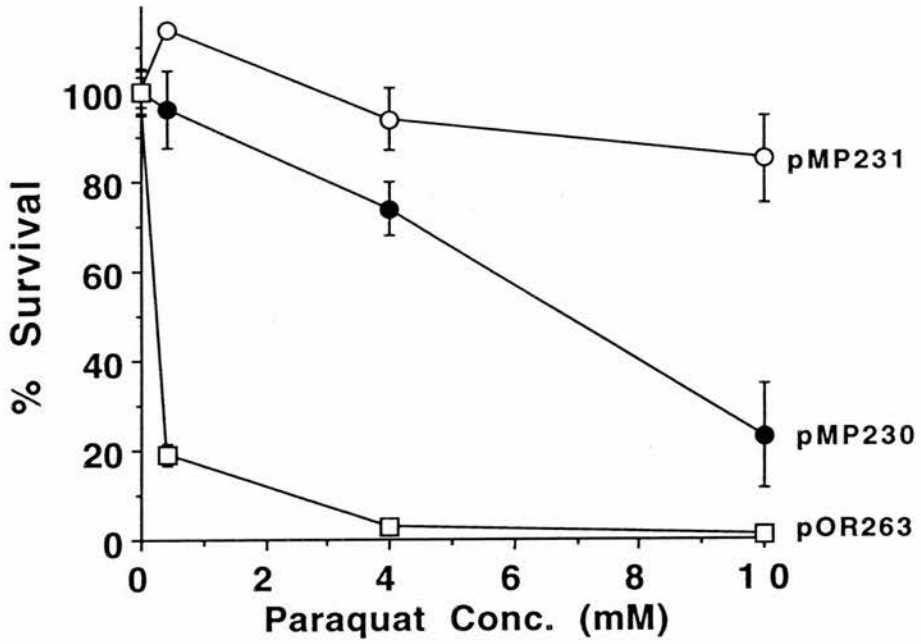



**Figure 3.6. Survival of S. typhimurium (TA1535) transformed with pMP231 (control) and the rat reductase expression vectors pMP230 and pOR263 following exposure to paraquat.** All measurements are in triplicate with S.D.s shown.

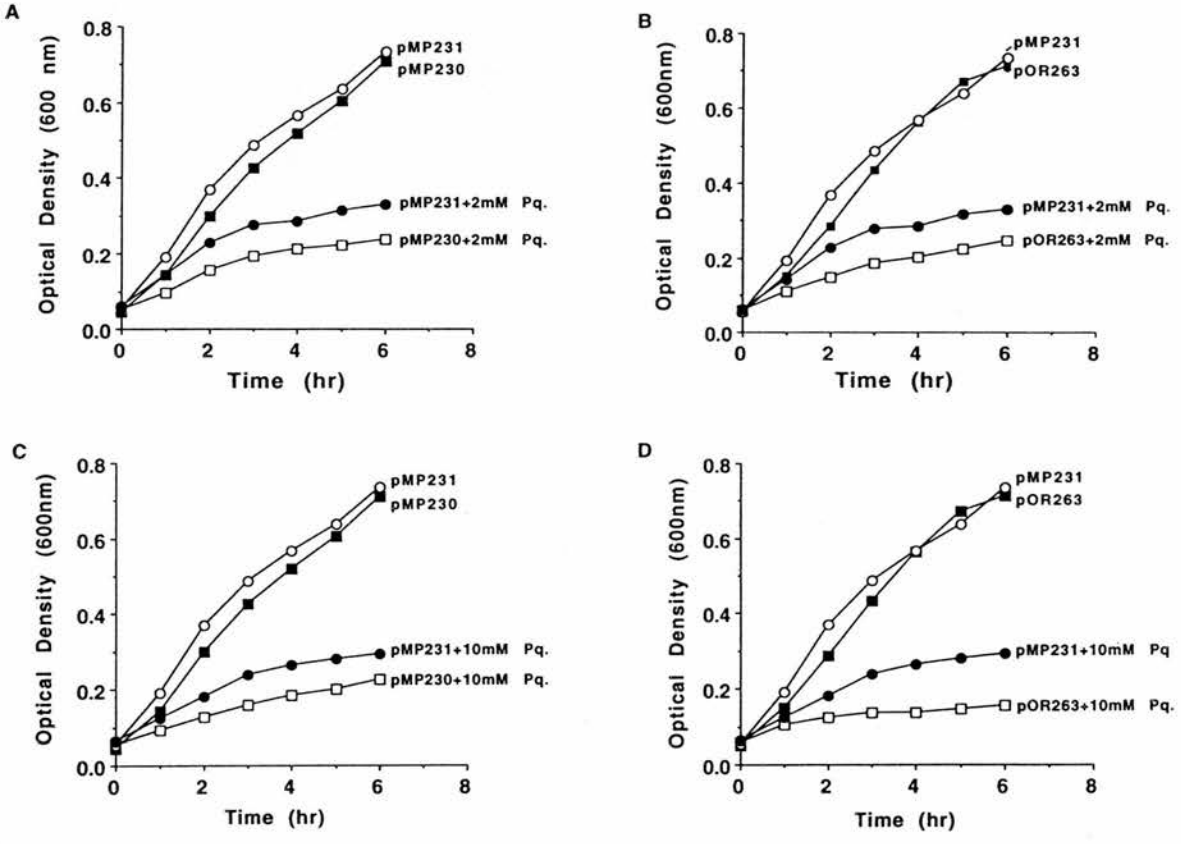



**Figure 3.7. Effect of paraquat on the growth of S.typhimurium (TA1535).** (A,C) pMP231 (control) and the rat reductase expression vector pMP230. (B,D) pMP231 and the rat reductase expression vector pOR263. Paraquat (pq) concentrations are shown.

shown in Figure 3.6. It can be clearly seen that the P450 reductase expressing bacteria are more susceptible to the cytotoxic effects of paraquat in comparison to the non-expressing strain. This appears to be dose-dependent and also more enhanced in the bacteria expressing the greater levels of P450 reductase (pOR263 compared with pMP230). The ability of P450 reductase to affect the growth of TA1535 in liquid culture is shown in Figure 3.7. Again the pOR263 harbouring strain appears most sensitive to the effects of paraquat with almost complete inhibition of growth seen in this strain at a concentration of 10mM paraquat. These results clearly establish that P450 reductase has an enhanced effect on the cytotoxic and growth inhibitory effects of paraquat in *S. typhimurium*.

### 3.3.2 Cytotoxic and growth inhibitory effects of menadione on P450 reductase expressing and non-expressing *S. typhimurium*.

Menadione is an electrophilic quinone compound (Figure 3.5) which is easily reduced to the semiquinone form which in turn readily reduces $O_2$ to $O_2^-$. Menadione is able to be reduced by P450 reductase *in vitro* (Vermillion and Coon, 1978a). Figure 3.8 shows that expression of P450 reductase in *S. typhimurium* (using both pOR263, pMP230) enhances the cytotoxic effect of the quinone containing compound menadione when compared to the non-expressing strain (pMP231). In this case there appeared to be little difference between the greater P450 reductase expressing (pOR263) and the lower expressing P450 reductase (pMP230) strains. However, when looking at the ability of menadione to inhibit the growth of *S.typhimurium* in liquid culture (Figure 3.9) the effects of P450 reductase expression were dramatic in the pOR263 harbouring strain. Expression of P450 reductase from the pMP230 vector appeared to have little effect compared to the control. Strains were checked for their ability to reduce cytochrome c and the pMP230 harbouring strain appeared to still have cytochrome c reductase activity. One reason for the difference in effect seen between the two P450 reductase expressing bacteria maybe the localisation of the P450 reductase in the bacteria. The pOR263 vector directs the expressed P450 reductase to the periplasm through the incorporation of the ompA signal peptide sequence to the amino-terminus of the protein. Therefore, the dramatic effect seen for the pOR263 harbouring strain maybe due to the membrane localisation of P450 reductase.

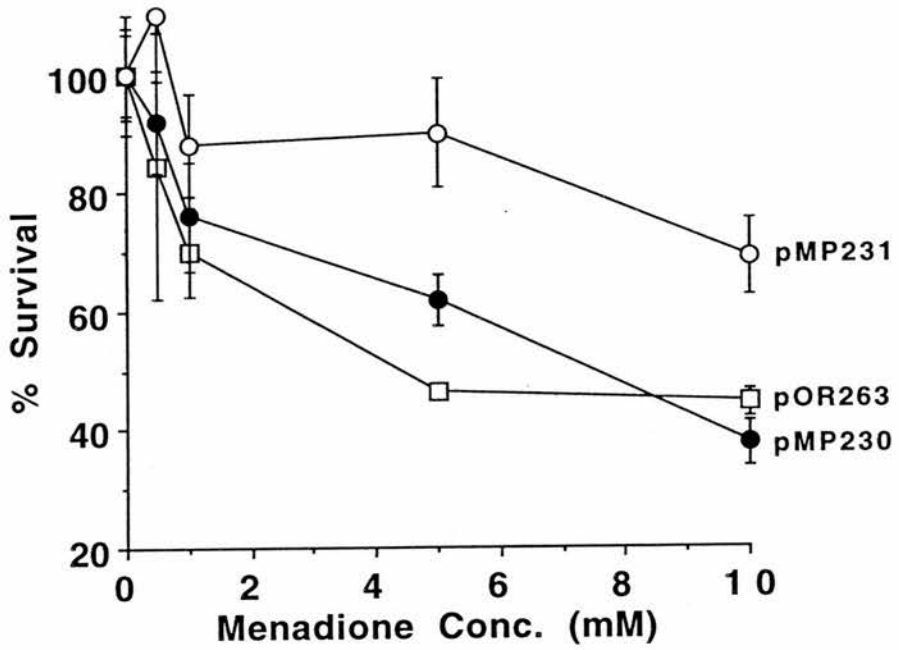



**Figure 3.8. Survival of S. typhimurium (TA1535) transformed with pMP231 (control) and the rat reductase expression vectors pMP230 and pOR263 following exposure to menadione.** All measurements are in triplicate with S.D.s shown.

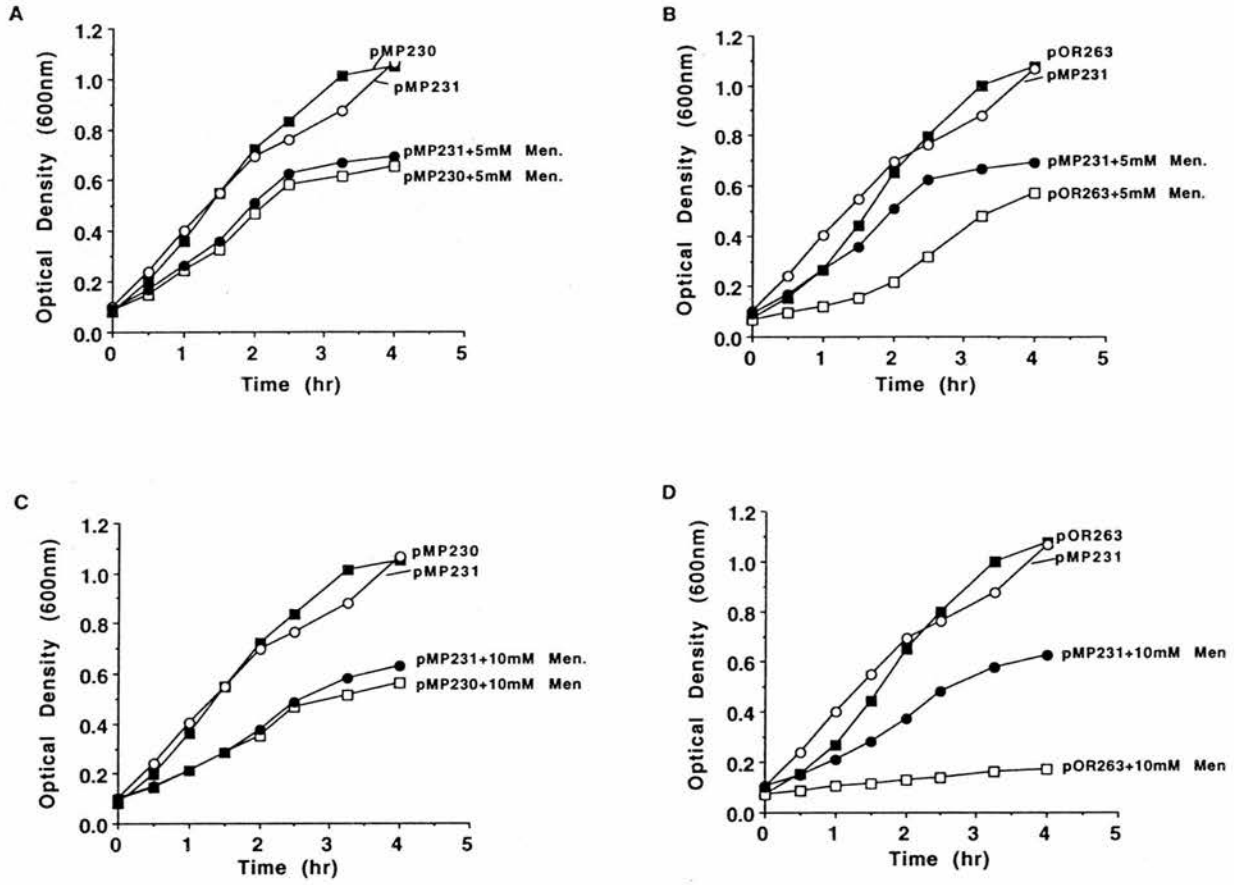



**Figure 3.9. Effect of menadione on the growth of S.typhimurium (TA1535).** (A,C) pMP231 (control) and the rat reductase expression vector pMP230. (B,D) pMP231 and the rat reductase expression vector pOR263. Menadione (men.) concentrations are shown.

### 3.3.2 Cytotoxic and growth inhibitory effects of doxorubicin on P450 reductase expressing and non-expressing *S. typhimurium*.

Doxorubicin is an anthracyclin anti-cancer drug (Figure 3.5) and one of the most valuable of this class of drugs in present clinical use. A single explanation for its anti-tumour activity (if indeed one exists) has proved elusive despite over 20 years of research. However, four mechanisms which could produce tumour cell cytotoxicity have emerged. These are a) DNA intercalation and stabilisation of the topoisomerase II and DNA cleavage complex (Liu *et al.*, 1989); b) perturbation of the biochemistry of the cell membrane (Tritton and Yee, 1982); c) covalent binding to DNA (Moore, 1977 and Sinha and Gregory, 1981) and d) enzyme catalysed and iron-mediated free radical formation (Handa and Sato , 1975 and Powis, 1987). At present, the literature favours the first of these mechanisms. However, the final mechanism is obviously of relevance to the work described here. Handa and Sato (1975) first showed *in vitro* that doxorubicin could be converted to a semiquinone free radical by an NADPH dependent one electron reduction. This or a similar process was also believed to occur in intact Ehrlich ascites cells. No specific enzyme has been discovered which acts as a doxorubicin quinone reductase. However, several enzymes accept the drug as a substrate for one electron reduction *in vitro* including P450 reductase, NADH dehydrogenase, xanthine oxidase and an unknown component of the nuclei (Cummings *et al.*, 1991). The cytotoxic effects of various concentrations of doxorubicin towards the P450 reductase expressing and non-expressing *S.typhimurium* are shown in Figure 3.10. It can be seen that there is no significant difference between the P450 reductase expressing and non-expressing bacteria. The data shown in Figure 3.10 suggests that P450 reductase has no effect on the cytotoxic effects of doxorubicin on *S.typhimurium*. This suggests that free radical formation may not play a role in the cytotoxicity observed in this system.

The effects of doxorubicin on the growth of *S. typhimurium* in liquid culture are shown in Figures 3.11. Apart from Figure 3.11D, it appears that doxorubicin has little effect on growth inhibition of either expressing or non-expressing *S.typhimurium*. One problem in the experiments described here is that it is unknown how much of the doxorubicin is being taken up by these bacteria, if at all. The latter experiments were carried out in liquid culture

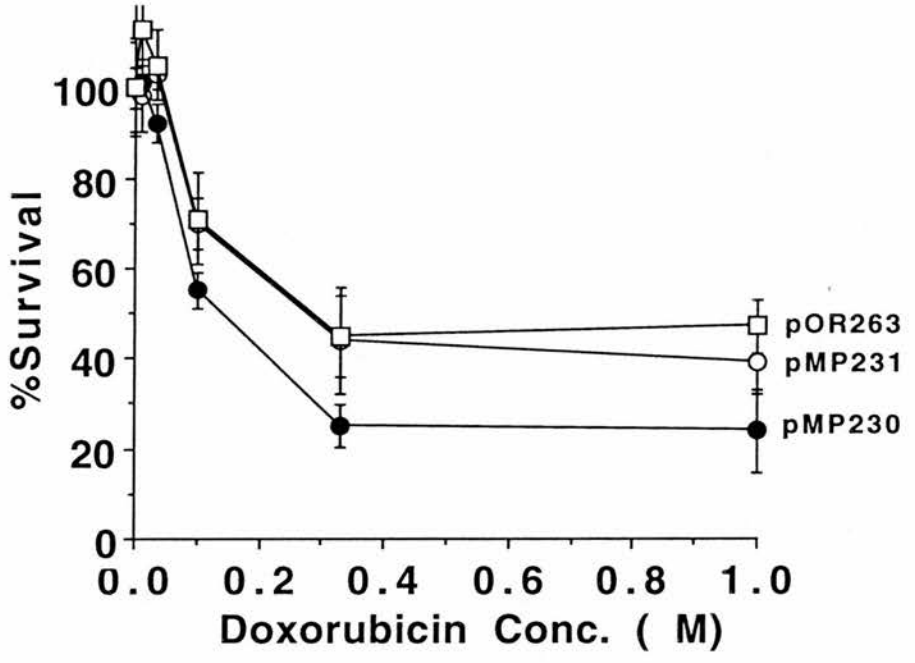



**Figure 3.10. Survival of S. typhimurium (TA1535) transformed with pMP231 (control) and the rat reductase expression vectors pMP230 and pOR263 following exposure to doxorubicin.** All measurements are in triplicate with S.D.s shown.

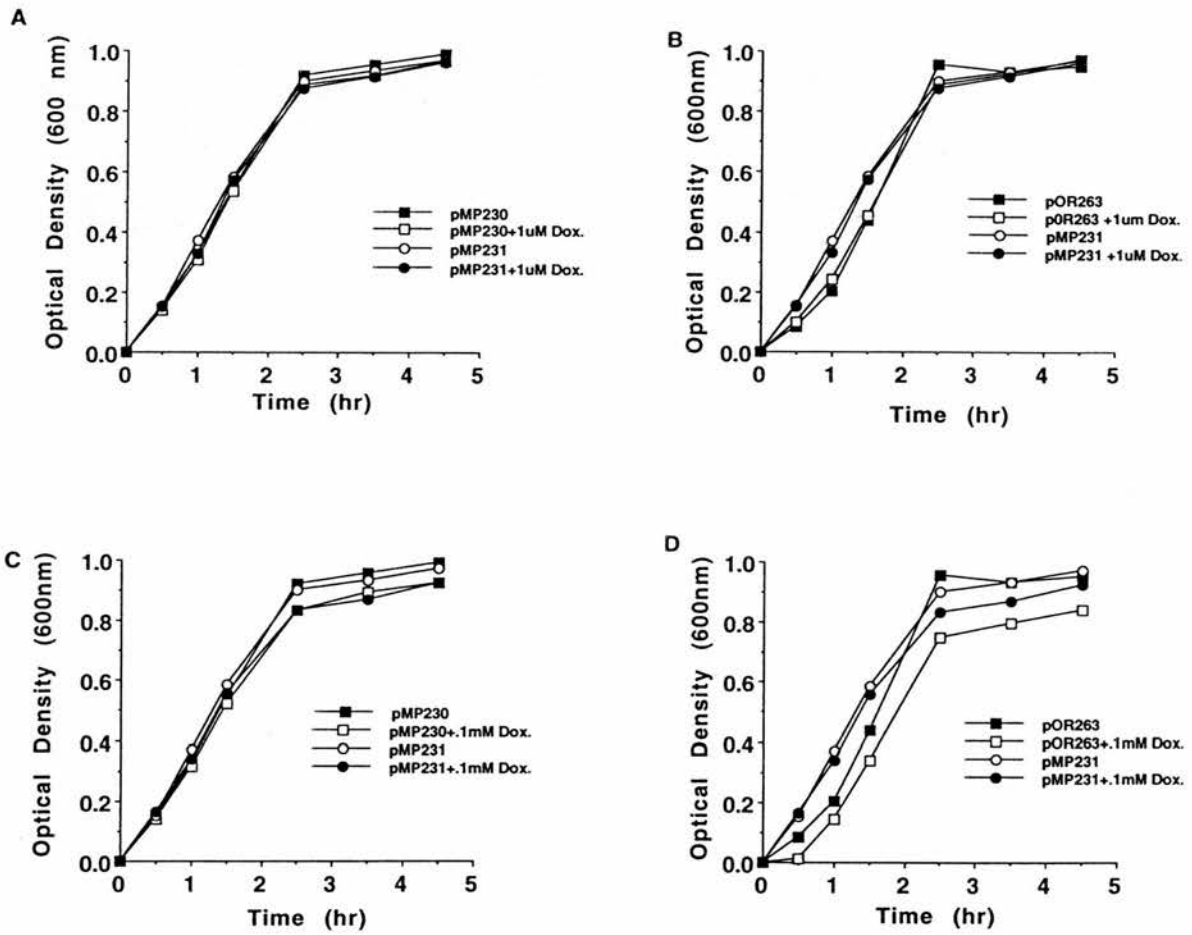



**Figure 3.11. Effect of doxorubicin on the growth of S.typhimurium (TA1535).** (A,C) pMP231 (control) and the rat reductase expression vector pMP230. (B,D) pMP231 and the rat reductase expression vector pOR263. Doxorubicin (dox.) concentrations are shown.

medium (nutrient broth) whereas the cytotoxicity experiment was carried out by treating cells with drug in phosphate buffered saline.

For free radical formation by doxorubicin to have any clinical relevence then a variety of criteria would have to be fulfilled such as; a) identification of hydroxyl radicals being produced in a site specific manner; b) detection of damage consistent with free radical formation and c) antagonism of cytotoxicity by free radical scavengers and detoxification enzymes (Cummings *et al.*, 1991). Evidence for the above is not very convincing. For example, $OH^{\cdot}$ radicals have been detected by electron spin resonance, however, these were observed after treatment of MCF7 cells with a doxrubicin concentration two orders of magnitude higher than clinically achievable concentrations and the majority of these radicals were detected outside the cells (Sinha *et al.*, 1987). Another study failed to detect $OH^{\cdot}$ in MCF7 cells and showed $O_2^{\cdot-}$ to be produced in the extracellular space after diffusion of the doxorubicin semiquinone from inside the cells (Alegria *et al.*, 1989). Non-protein associated DNA breaks, consistent with free radical damage have only been observed at concentrations well in excess of the IC50 (Potmisil *et al.*, 1983). In certain cell lines (MCF7 and Ehrlich ascites) free radical scavangers antagonise cytotoxicity while in other cell systems (A2780) they do not (Keizer, 1990).

### 3.4 Effect of redox cycling agents on enzyme levels controlled by the *sox RS* and *OxyR* regulons in *E.coli*.

Oxidative stress exerted by superoxide generating agents such as paraquat triggers the *sox RS* regulon of *E.coli*. (Demple and Amabile-Cuevos, 1991). In this system, sox R protein is the redox sensitive activator of the *sox S* gene. The sox S protein activates around 10 promoters of this regulon. Proteins induced by this system include Mn and Fe superoxide dismuthases, glucose-6-phosphate dehydrogenase (G6PDH), endonuclease IV and NADH dehydrogenase These proteins can be thought of as being induced to play a defensive role against the adverse effects of oxygen free radicals.

The oxyR protein is the transcriptional activator of an $H_2O_2$ inducible regulon (Stortz *et al.*, 1991). The oxyR protein is only transcriptionally active when oxidised and turns on the expression of catalase, glutathione reductase and alkyl hydroperoxide reductase. Both the *OxyR* and *SoxRS* regulons have



| | | *SoxRS* | | *OxyR* | |
|---|---|---|---|---|---|
| | PQ. (mM) | SOD (units/mg) | | Catalase (units/mg) | |
| pMP231 | 0 | 29.8 | (100%) | 28.9 | (100%) |
| | 2 | 233.9 | (785%) | 79.5 | (275%) |
| | 10 | 249.8 | (838%) | 88.9 | (307%) |
| pOR263 | 0 | 41.6 | (100%) | 30.8 | (100%) |
| | 2 | 313.3 | (753%) | 130.1 | (422%) |
| | 10 | 287.3 | (690%) | 151.0 | (490%) |
| pMP230 | 0 | 35.1 | (100%) | 33.3 | (100%) |
| | 2 | 300.0 | (855%) | 69.5 | (209%) |
| | 10 | 255.1 | (727%) | 70.2 | (211%) |

**Table 3.1. Effect of paraquat on enzyme levels controlled by the *soxRS* and *oxyR* regulons in *E.coli*.**

| | | *SoxRS* | | *OxyR* | |
|---|---|---|---|---|---|
| | Men. (mM) | SOD (units/mg) | | Catalase (units/mg) | |
| pMP231 | 0 | 27.5 | (100%) | 25.4 | (100%) |
| | 0.5 | 81.9 | (298%) | 115.4 | (454%) |
| | 10 | 72.8 | (265%) | 102.3 | (402%) |
| pOR263 | 0 | 26.6 | (100%) | 33.3 | (100%) |
| | 0.5 | 69.4 | (261%) | 88.2 | (265%) |
| | 10 | 71.2 | (268%) | 87.5 | (263%) |
| pMP230 | 0 | 27.3 | (100%) | 33.8 | (100%) |
| | 0.5 | 56.0 | (205%) | 107.7 | (319%) |
| | 10 | 53.1 | (195%) | 67.7 | (200%) |

**Table 3.2. Effect of menadione on enzyme levels controlled by the *soxRS* and *oxyR* regulons in *E.coli*.**

| | | *SoxRS* | | *OxyR* | |
|---|---|---|---|---|---|
| | Dox. (μM) | SOD (units/mg) | | Catalase (units/mg) | |
| pMP231 | 0 | 34.4 | (100%) | 27.8 | (100%) |
| | 1 | 26.0 | (75%) | 17.0 | (61%) |
| | 100 | 17.0 | (49%) | 18.6 | (67%) |
| pOR263 | 0 | 33.6 | (100%) | 23.8 | (100%) |
| | 1 | 29.8 | (89%) | 19.6 | (82%) |
| | 100 | 29.3 | (87%) | 17.9 | (75%) |
| pMP230 | 0 | 21.7 | (100%) | 22.1 | (100%) |
| | 1 | 27.7 | (78.3%) | 15.3 | (69%) |
| | 100 | 20.2 | (93%) | 18.9 | (86%) |

**Table 3.3. Effect of doxorubicin on enzyme levels controlled by the *soxRS* and *oxyR* regulons in *E.coli*.**

been more fully characterised in *E.coli* than in *S.typhimurium* (Farr and Kogoma, 1991).

The effect of P450 reductase expression on these regulons was investigated in the presence and absence of a redox cycling compound. *E.coli* were grown in the presence of the chemical and after 4 hours extracts were prepared and assays performed on the soluble fraction for superoxide dismuthase and catalase activities. Effects of paraquat, menadione and doxorubicin on these enzyme levels are shown in Tables 3.1, 3.2 and 3.3 respectively. What can be seen from this data is that P450 reductase expressing bacteria had a variety of different effects on the enzyme levels compared to non-expressing *E.coli*. An important observation is that the regulons appeared not to be induced by P450 reductase expressing bacteria in the absence of a redox cycling compound. This suggests that if any free radicals are being produced by the expresed reductase they are not in sufficient concentration to ellicit a response. That doxorubicin did not induce any of the protective enzymes suggests that if redox cycling of this drug was occurring in this system then it was not at a level high enough to induce a response.

### 3.5 Summary.

The data in this chapter has served to highlight the use of P450 reductase *S. typhimurium* strains in the study of *in vivo* activation of redox cycling compounds. Two well known redox cycling compounds, paraquat and menadione had dramatic effects on the bacteria expressing P450 reductase. However, doxorubicin had no clear effect over wild type. This system may be of value in the study of the bioactivation of proposed redox cycling compounds. However, further studies using other compounds will need to be looked at.

The work on the *Sox RS* and *Oxy R* regulons in *E. coli* showed that when P450 reductase expressing bacteria were subjected to the compound of interest there was little effect over wild type levels of enzymes regulated by these systems. However, of importance from this work is the fact that P450 reductase expressing bacteria, in the absence of any redox cycling compound did not induce a stress response via these regulons. Since heterologous expression of mammalian proteins is becoming ever important for the pharmaceutical/ biotechnology industries, it is important to know the stresses imposed on host cells by the foreign protein.

# Chapter 4. Expression, purification and biochemical characterisation of domains of NADPH-Cytochrome P450 oxidoreductase.

## 4.1 Introduction and Aims.

From the original sequence alignments of the deduced amino-acid sequence from the rat P450red cDNA (Porter and Kasper, 1985; Porter and Kasper, 1986; Porter *et al.*, 1990) it was evident that P450 reductase contained two separate and distinct flavin binding domains. The two flavin binding domains found in P450 reductase were proposed to have been the result of a fusion of two ancestral flavoproteins rather than the outcome of a gene duplication event of a single flavin binding domain (Porter and Kasper, 1985). Porter and Kasper (1986) went on to propose that the fusion was of two ancient proteins that lay in tandem on a primordial operon.

These workers found that the N-terminal portion of the protein shows strong homology with the bacterial flavodoxin from *Desulfovibrio vulgaris*. The C-terminal portion of the protein shows homology with spinach ferredoxin $NADP^+$ reductase (FNR). Figures 4.1A,B,C show the results of multiple sequence alignments carried out using the programs 'Pileup' and 'Prettybox' from the GCG computer package. These Figures highlight the regions of importance in flavin binding and NADPH binding as deduced previously from the known crystal structures of flavodoxin, from *Desulfovibrio vulgaris*, and ferredoxin $NADP^+$ reductase, from spinach (Porter and Kasper, 1985, Porter and Kasper, 1986, Porter *et al.*, 1990, Karplus *et al.*, 1991, Shen and Kasper, 1993). In Figure 4.1B, regions involved in binding the FMN phosphate group and the FMN isoalloxazine ring are shown. In Figure 4.1C regions involved in binding the FAD pyrophosphate group and isoalloxazine ring are shown along with a region at the C-termini shown to interact with the dimethylbenzene ring of FAD. Two regions proposed to be involved in NADPH binding are illustrated in Figure 4.1C. NADPH 1 shows the dinucleotide binding site while NADPH 2 is a region proposed to be involved in binding the 2' phosphate of NADPH. The amino-acid sequences of P450red from *Saccharomyces cerivisiae* and *Bacillus megaterium* along with human brain nitric oxide synthase (NOS) and sulphite reductase from *Salmonella typhimurium* are included to show the striking homology across members of the FMN/FAD containing family of flavoproteins. Regions of P450red

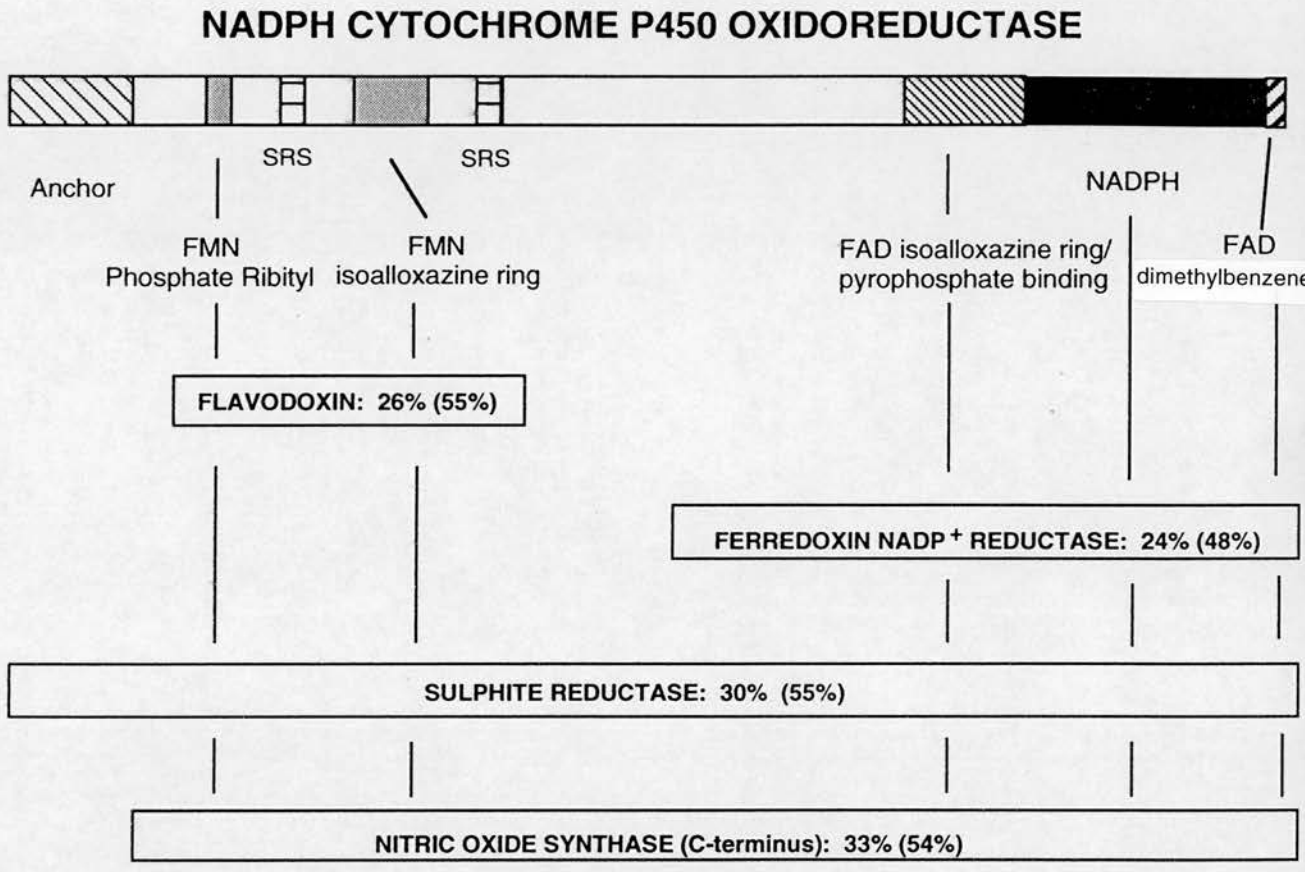



**Figure 4.1A** Summary diagram of amino-acid sequence alignments of P450 reductase with *desulfovibrio vulgaris* flavodoxin; spinach ferredoxin NADP+ reductase; *salmonella typhimurium* sulfite reductase; human brain nitric oxide synthase (C-terminal half). Percentage identities are shown with homologies in brackets. Regions involved in FMN and FAD/NADPH binding are shown, as deduced from the crystal structures of flavodoxin and ferredoxin NADP+ reductase respectively. Alignments were performed using the 'Bestfit' program.

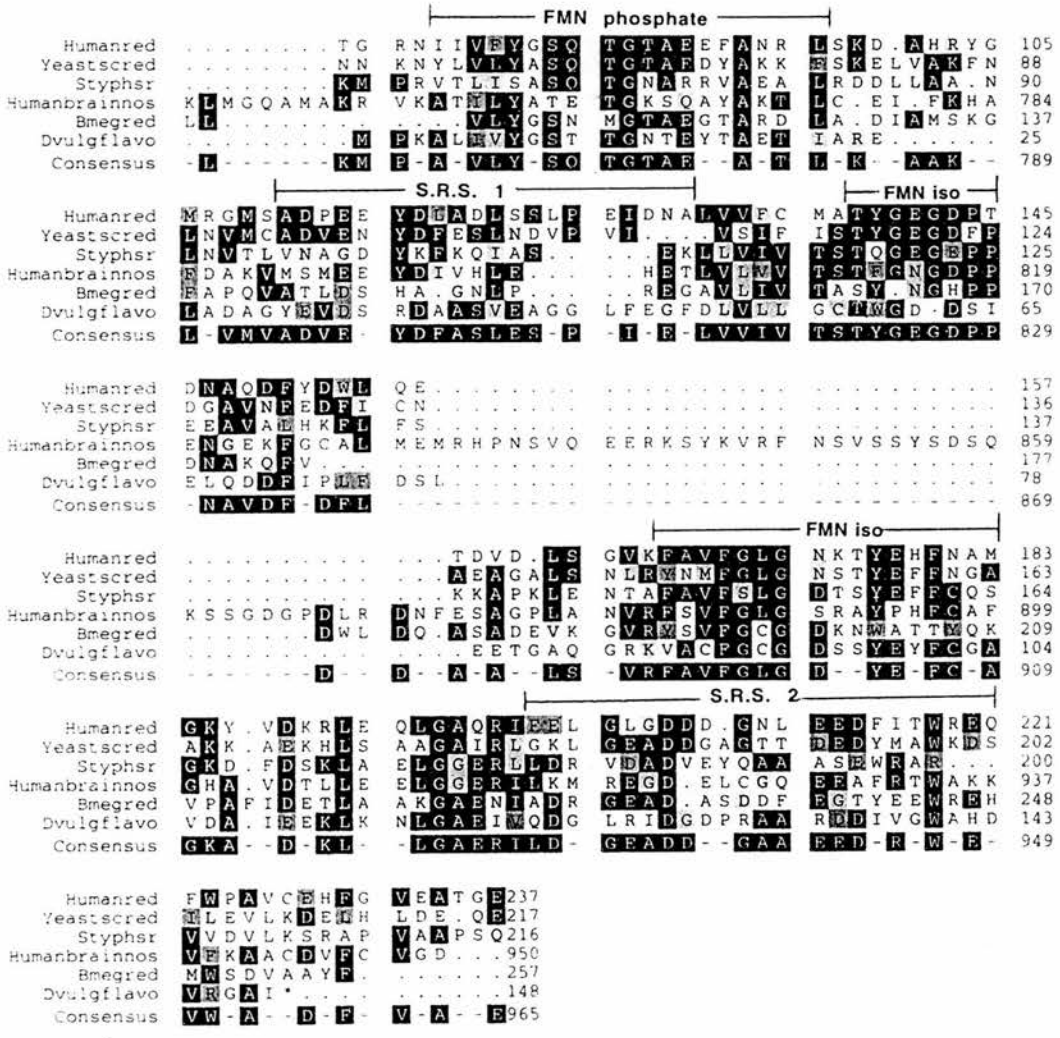



**Figure 4.1B** Multiple amino acid sequence alignment of; P450 reductases from human, *bacillus megaterium* and *sacharomyces cerivisiae*; *Salmonella typhimurium* sulphite reductase; human brain nitric oxide synthase and *desulfovibrio vulgaris* flavodoxin. The alignment was performed using 'Pileup' and 'Prettybox' from the GCG package. Residues in bold are identical while those shaded are conserved. Regions shown are discussed in the text. iso, isoalloxazine;. SRS, substrate recognition site. Amino-acid numbers are shown.

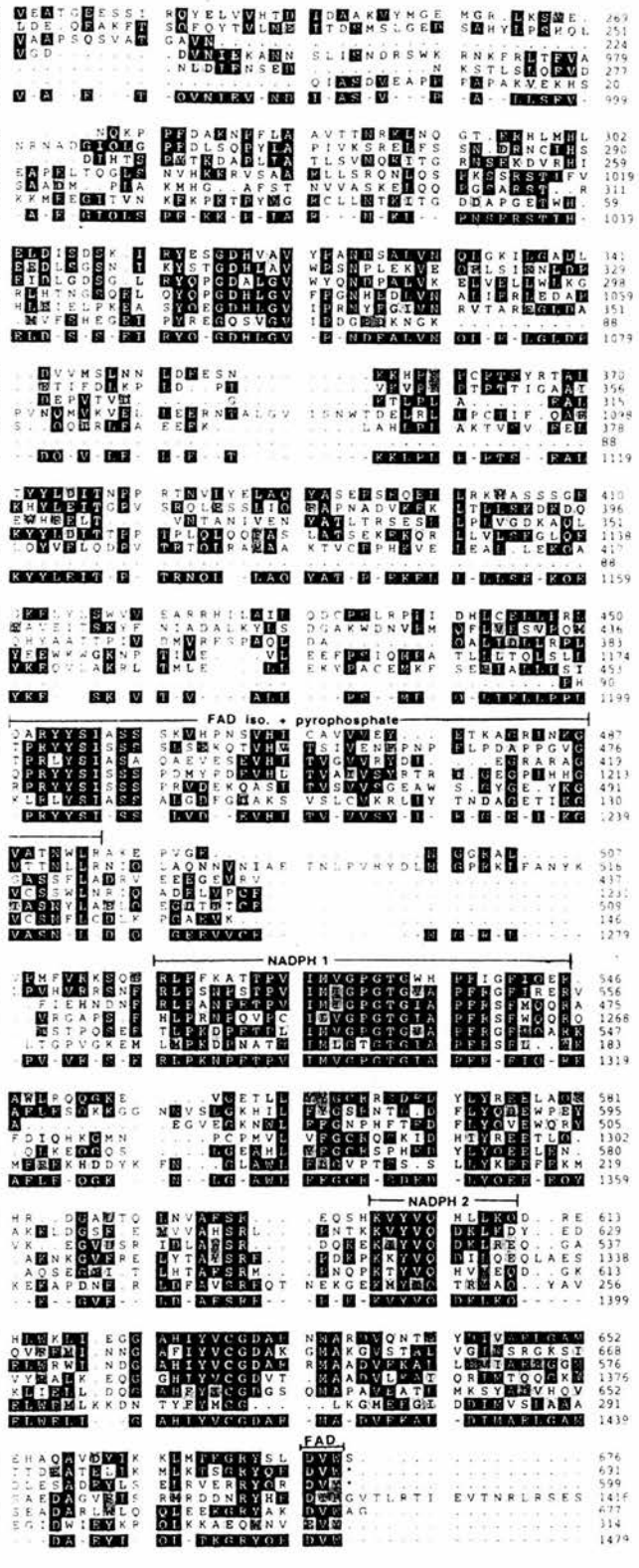



**Figure 4.1C** Multiple amino acid sequence alignment of; P450 reductases from human, *bacillus megaterium* and *sacharomyces cerivisiae*; *Salmonella typhimurium* sulphite reductase; human brain nitric oxide synthase and spinach NADP+ ferredoxin reductase. This alignment was performed using 'Pileup' and 'Prettybox' from the GCG package. Residues in bold are identical while those shaded are conserved. Regions shown are discussed in the text. iso, isoalloxazine. Amino-acid numbers are shown..

proposed to be involved in P450 (SRS) and cytochrome c (SRS 2) interactions are also shown in Figure 4.1B (Nadler and Strobel, 1992, Nisimoto, 1986).

Work on the structural analysis of P450 reductase has so far focused on the use of site-directed-mutagenesis and chemical modification to alter proposed residues of structural importance (Shen *et al.*, 1989; Shen and Kasper, 1990; Sem and Kasper, 1993; Nisimoto and Shibata, 1982; Lazar *et al*, 1977, Vogel and Lumper, 1986; Haniu *et al.*, 1984, 1989, Nisimoto, 1986). The residues for the site-directed mutagenesis analysis were deduced from the alignments with the aforementioned bacterial and plant proteins for which the crystal structures are known. For example, site-directed mutagenesis of the rat protein has demonstrated the roles of Tyr 140 and Tyr 178 in FMN binding and catalysis (Shen *et al.*, 1989). While Arg 597 of the rat reductase has been shown to be of importance in discriminating between NADH and NADPH (Sem and Kasper, 1993). Site-directed mutagensis of acidic residues in the region 200-220 of the rat protein has provided some evidence for their interaction with cytochrome c and P450 (Shen and Kasper, 1990). Cross linking studies have also shown the importance of this region in cytochrome c binding (Nisimoto, 1986)

However, no work has so far looked at dissecting P450 reductase or structurally similar proteins into the distinct functional domains that are suggested from the sequence alignments. One reason for believing that splitting the protein up into distinct flavin binding units could be successful is that the proposed contact points for FMN and FAD binding are in different regions of the protein as suggested from the proposed evolution of the protein. An argument against the ability to dissect the protein is that co-operativity between the flavin binding domains may be of importance for their correct folding and hence incorporation of their respective flavins (Vermillion and Coon, 1978b, 1981; Iyanagi *et al.*, 1981; Kurzban and Strobel, 1986, Kurzban *et al.*, 1990). That proteins can be split into functional domains is exemplified by the steroid hormone binding receptors (Kumar *et al.*, 1986, 1987, Green and Chambon, 1987) and tissue plasminogen activator (Patthy, 1985). The latter protein being the first example of what is termed a 'mosaic' protein. These 'mosaic' proteins have a modular structure in a manner similar to that proposed for P450 reductase. These proteins are totally dissimilar but the modular structure of the proteins serves a point, *i.e.* one region serves one function while another quite separate region of the protein serves another. Using the steroid hormone binding receptors as an example, one region of the

protein acts as the hormonal receptor while the other binds DNA (Kumer *et al.*, 1986, 1987; Green and Chambon, 1987). P450 reductase binds three different prosthetic groups, namely NADPH, FMN, FAD, and has the ability to interact with both membranes and with cytochromes P450. This would suggest the existence of distinct functional domains in this protein.

In order to attempt the dissection of P450 reductase into distinct functional domains a variety of regions of the protein were proposed for expression in *E.coli* (Figure 4.2). Bacterial expression of these proposed domains would not only allow insights into the basic structure, function and evolution of the protein but provide an abundant source of material for future structural analysis, such as NMR and X-ray crystallography. Analysis of the rat P450 reductase gene by Porter *et al.*, (1990) revealed that distinct groups of exons coded for the proposed functional domains of the protein as shown in Figure 4.2. The regions of the human P450 reductase to be used for bacterial expression were decided upon using the organisation of the rat gene as a model. The rat gene was used as a model since the human gene for P450 reductase has not yet been cloned. The rat and human reductases show 97% similarity and 92% identity at the amino acid level so it was felt that the rat gene would be a representative model to define the functional units of the human protein using exon/intron boundaries (Figure 4.2). Indeed, intron/exon boundaries have been shown to be identical for a variety of human and rat genes including the human and rat UDPGT genes (Monaghaun, 1994) the cytochrome P450 genes in family 2 (Hawkins, 1988) and the aldolase genes (Rottman *et al.*, 1987).

The extensive analysis of gene structure carried out by Traut (1988) showed that, for the genes he analysed, there was a greater than 50% correlation between exons and structural/functional domains of the protein. Some examples where exons have been shown to correlate with structural/functional domains include glyceraldehyde phosphate dehydrogenase (Shih *et al.*, 1988), triosephosphate dehydrogenase (Straus and Gilbert, 1985), immunoglobulin G heavy chain (Sakano *et al.*, 1979) and lysozyme (Jong *et al.*, 1980). Some examples where exons have been shown not to correlate with structural/functional units of the protein include carboxypeptidase (Quinto *et al.*, 1982), lactate dehydrogenase (Li *et al.*, 1982) calmodulin (Simmen *et al.*, 1985) and actin (Weber and Kabsch, 1994). As can be imagined, from the above analysis of protein structure with exon/intron organisation, there has been much debate in the literature on whether

proteins have evolved through the assembly of exons that define distinct structural/functional units or not. This debate ensued after the observation that eukaryotic genes are generally built from short exons separated by long introns (reviewed by Witkowski, 1988). The opposing viewpoints are based on the models termed 'intron early' and 'intron late'. In the intron early model, genes were assembled in early evolution from exons encoding functional domains, folding regions or structural elements via intron mediated recombinations thus allowing for the production of proteins with a variety of functions (Gilbert, 1988). This model assumes a complete loss of introns in prokaryotes while in eukaryotes introns are thought of as primitive relics which were in part lost and part retained (Gilbert, 1978; Cornish-Bowden, 1985). In contrast, the intron late model assumes that introns were gained only by early eukaryotic genomes and during their later evolution *i.e.* introns invaded the otherwise uninterupted coding regions of genes (Cavallier-Smith, 1985). However, an important aspect of both models is that they allow for exon shuffling later in eukaryotic evolution. Which model P450 reductase supports is unclear since both have their merits. Specific exons of the rat reductase gene code for the proposed prosthetic group binding sites while other important binding sites are split between exons (Porter *et al.*, 1990). P450 reductase can be thought of as an ancient protein since it exists as a fusion with a P450 in the bacteria *Bacillus megaterium* (ATCC 14481) (Nahri and Fulco, 1986). The ancestral reductase protein may have lost introns during the evolution of *B. megaterium* or may have gained introns during the evolution of the eukaryotic reductases.

To define the regions of P450 reductase to be expressed on the intron/exon organistion of the gene may be futile since the original structural boundaries, that may or may not have existed, in the ancestral reductase will most probably have been lost through the further evolution of the protein in the modern forms of the reductase. P450 reductase has evolved to serve a completely different function from that of its proposed ancestors, the flavodoxin and ferredoxin $NADP^+$ reductase. However, using the intron/exon boundaries will, at the least, be useful for initial studies in defining the limits of the proposed functional domains not only in P450 reductase but in other structurally related proteins such as the nitric oxide synthases and sulfite reductases.

The initial aims of this work were to produce functional domains of human P450 reductase, as proposed by Porter and Kasper (1986), using a bacterial

expression system. The domains for expression were, as discussed above, defined by the intron/exon boundary of the rat gene (Porter *et al.*, 1990) and shown in Figure 4.2. Why these domains were chosen will be discussed in the next section.

**4.2 /4.3 Results and discussion.**

## 4.2 Expression, purification and characterisation of the proposed functional domains of human NADPH cytochrome P450 oxidoreductase.

### 4.2.1. Proposed functional domains of human NADPH cytochrome P450 oxidoreductase used for bacterial expression.

A variety of domains of P450 reductase were chosen for expression (Figure 4.2) using the bacterial pET (plasmid for expression by T7 RNA polymerase) system (Figure 4.4). Exons encoding the domains for the proposed 'minimal' FMN and FAD/NADPH (termed FMN(small) and FAD/NADPH(small)) were selected for expression along with groups of exons that encoded regions outwith the minimally defined regions. Exons 4,5,6 contain all the contact points for FMN binding as defined by sequence alignment with the flavodoxin from *Desulfovibrio vulgaris* whose crystal structure is known (Porter and Kasper, 1986; Watenpaugh, 1973). This region was named FMN (small) while the other FMN regions contained exons 2-8 or exons 3-8 and are called the FMN/anchor and FMN domains respectively. Exons 12-16 were chosen for expression of the FAD/NADPH(small) domain and contained all the proposed contact points required for FAD and NADPH binding as defined by sequence alignment with spinach ferredoxin $NADP^+$ reductase whose crystal structure is known (Porter and Kasper, 1986; Karplus, 1991). Exons 8-16 were chosen for expression of a larger FAD/NADPH domain. The larger domains were chosen for expression since it was felt that regions outwith the so-called 'minimal' domains would be important in the overall folding of the domain to produce a functional unit. Although the most significant regions of alignment with the *Desulfovibrio vulgaris* flavodoxin and spinach FNR are within the 'minimal' domains, it must be remembered that P450 reductase has evolved to serve a different function than these two functionally dissimilar flavoproteins and hence P450 reductase may contain important

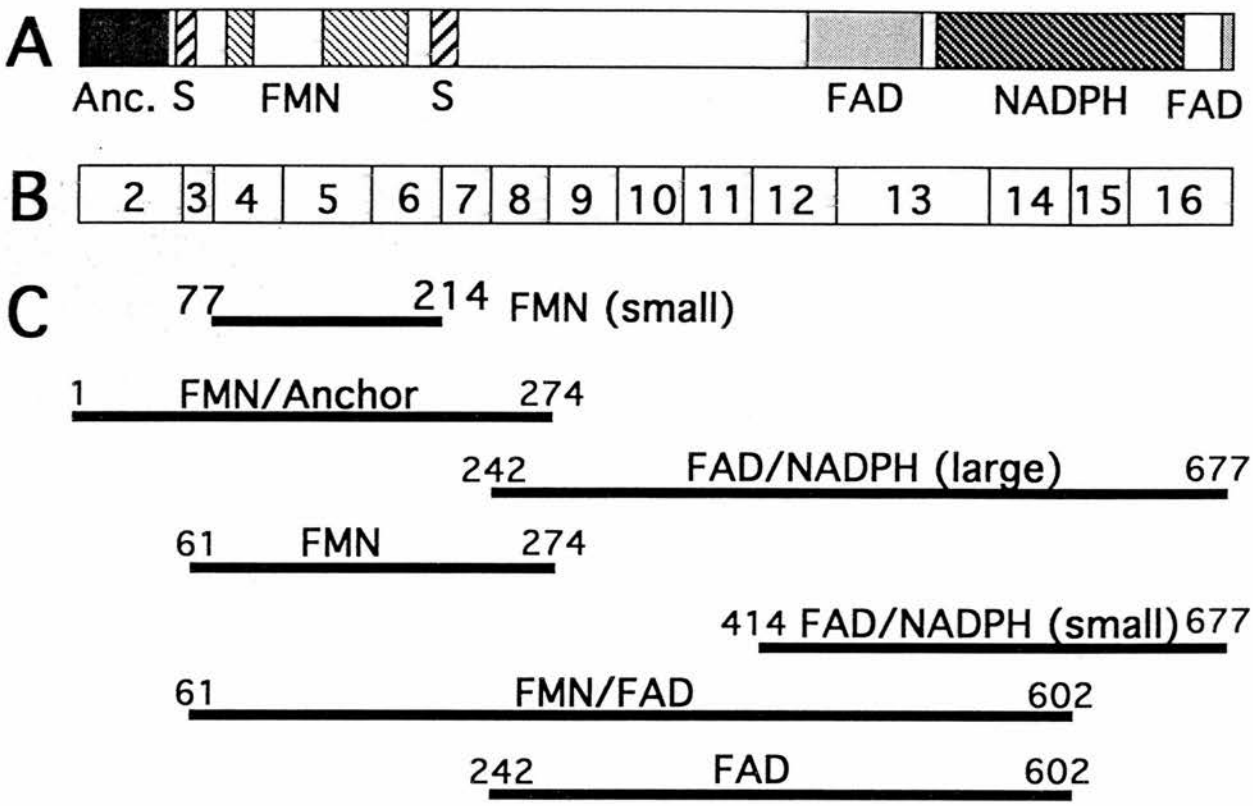



**Figure 4.2. Structure of P450 reductase and it putative domains. (A)** Organisation of the P450 reductase domains. Regions of structural importance are shown. Anc., membrane anchor region; S, substrate recognition site. (**B**) Exon organisation of the rat P450 reductase gene. (**C**) Domains made for expression in this study. Amino acids flanking the domains are shown. 5' oligonucleotides were derived from the start of the exons shown and incorporated an *Nde I* restriction site. 3' oligonucleotides were derived from the sequence at the ends of the exons and incorporated a stop codon followed by an *Xho I* restriction site.

regions for prosthetic group binding or protein folding outwith the 'minimal' regions described here. The FMN/FAD domain shown in Figure 4.2 contained all the proposed residues for FMN binding and the majority of residues important for FAD binding. However, this protein did not contain Tyr 677 and Glu 674 which, from alignment with spinach FNR, are proposed to be invloved in covering the face of the flavin and interacting with the dimethylbenzene ring of the flavin respectively (Karplus *et al.*, 1991). Also, this construct did not contain a significant portion of the NADPH binding region of the reductase (exons 15,16). The FAD domain (Figure 4.2) was similar to the large FAD/NADPH domain but did not contain the last two exons of the reductase, as described for the FMN/FAD domain.

The expression plasmid pET15b (Figure 4.4) utilises the immensly powerful bacteriophage T7 promoter along with T7 RNA polymerase. The pET system relies on there being a copy of T7 RNA polymerase incorporated into the genome of *E.coli*. (Studier and Moffat, 1986; Rosenberg *et al.* 1987). A copy of the polymerase has been established, under control of the *lacUV5* promoter, in the *E.coli* strain BL21(pLysS). On induction of BL21(pLysS) with IPTG the T7 RNA polymerase is produced. (N.B. Any leaked expression of T7 RNA polymerase before IPTG induction is inhibited by bacterial lysozyme which is present in the BL21 strain encoded by the plasmid pLysS). T7 RNA polymerase is highly specific for the T7 promoter found within pET15b and after its expression is induced it can lead to the majority of transcription within the *E.coli* being driven from the T7 promoter (Studier and Moffat, 1986; Rosenberg *et al.*; 1987). The ferocious nature of this system can however lead to the production of insoluble, unfolded protein termed 'inclusion bodies' (Williams *et al.*, 1982).

One major reason for chosing this plasmid was that by cloning into the unique *Nde* I and *Xho* I sites a 6x Histidine tag followed by a thrombin cleavage site would be engineered onto the $NH_2$-terminus of the expressed proteins (Figure 4.4). This would allow a single step purification using the affinity matrix nickel-agarose. Nickel-agarose chromatography also allows proteins that are insoluble to be purified under denaturing conditions.

### 4.2.2. Cloning of the human NADPH cytochrome P450 oxidoreductase cDNA.

The cDNA for the human reductase was cloned by PCR from human skin fibroblast cDNA using oligonucleotides for the 5' and 3' ends of the cDNA derived from the sequence of the human reductase (Shepherd *et al.*, 1990) as shown in Figure 4.3. The oligonucleotide for the 5' end had an *Eco RI* restriction site incorporated into it. After amplification (Figure 4.3), treatment with Klenow and digestion with *Eco RI*, the PCR products from two reactions were cloned into the *EcoRI/ Sma I* site of the phagemid pTZ19R. After sequence analysis it was found that the cDNA was identical to that of the human P450 reductase sequence present in the EMBL data-base (Shepherd *et al.*, 1992).

This cDNA was then used as the template for the amplification of the cDNAs encoding the domains of P450 reductase shown in Figure 4.2. The 5' oligonucleotides derived from the sequence at the start of the exons shown in Figure 4.2 incorporated an *Nde I* restriction site, while the 3' oligonucleotides derived from the sequence at the ends of the exons shown in Figure 4.2 incorporated a stop codon followed by an *Xho I* restriction site. After amplification the cDNAs were digested with *Nde I* and *Xho I* followed by ligation into the appropriate sites of the expression vector pET15b (Figure 4.4).

### 4.2.3. Expression of the proposed functional domains of human P450 oxidoreductase in *E.coli*.

Figures 4.5-4.11 show the expression and purification of the domains of P450 reductase that are illustrated in Figure 4.2 using the expression plasmid pET15b (Figure. 4.4). By cloning into the *Nde I/ XhoI* sites of pET15b a 6xHis-linker and thrombin cleavage site are engineered onto the $NH_2$-terminus of the expressed protein (Figure 4.4). The generation of a 6xHis linker on the $NH_2$-terminus of the expressed protein allows purification to be carried out employing nickel-agarose chromatography.

From the initial expression studies it became evident that the level of expression of soluble protein varied from one domain to the next. One problem of bacterial expression is the formation of inclusion bodies (Williams *et al.*, 1982). Inclusion bodies can be formed from the clumping together of proteins that are highly hydrophobic or proteins that do not fold correctly in

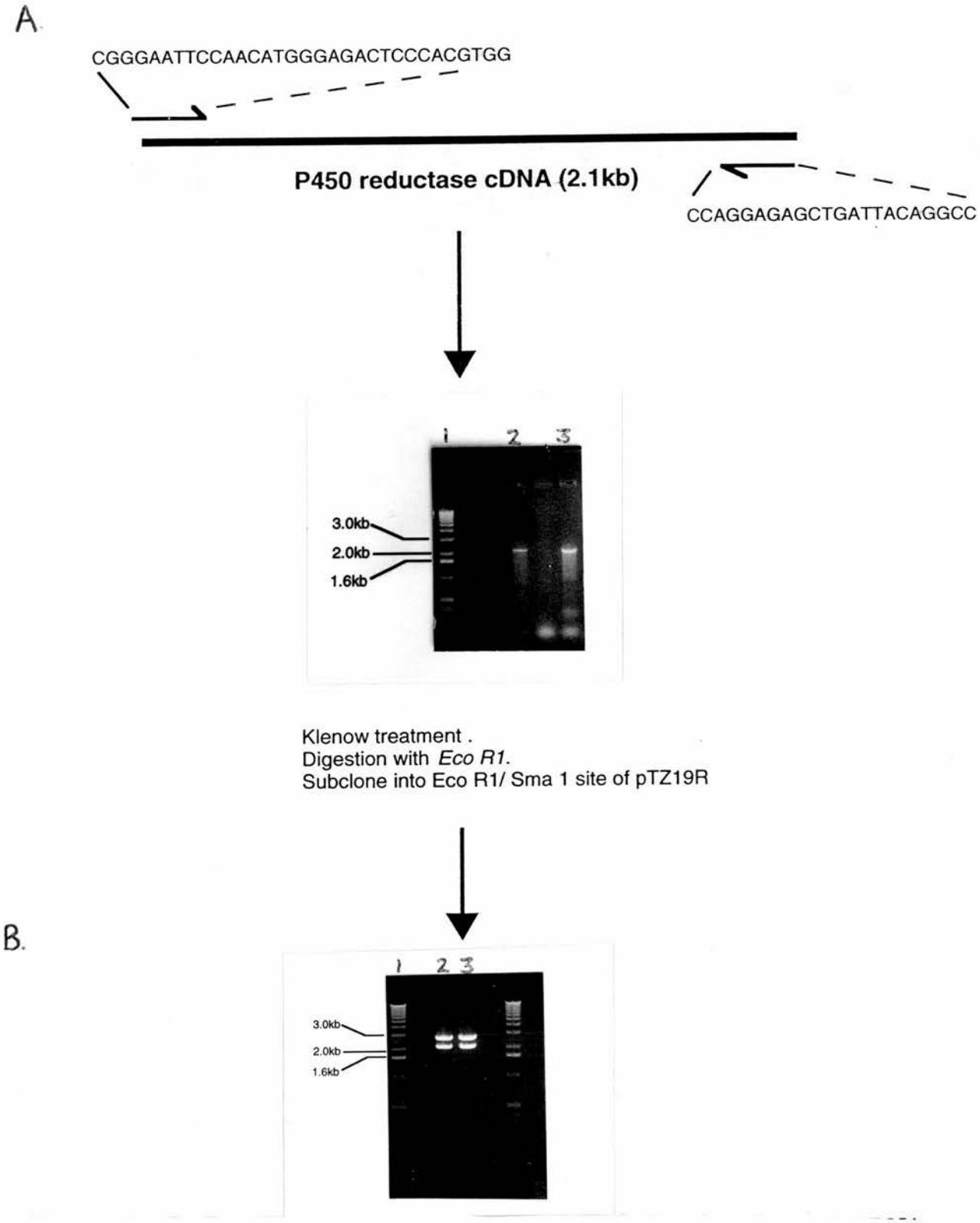



**Figure 4.3. Cloning of the human P450 reductase cDNA.** (A) The human cDNA was amplified by PCR (lanes 2,3) from skin fibroblast cDNA using the oligonucleotides shown. Lane 2; kb ladder. (B) The amplified 2.1 kb fragment was subcloned into the phagemid pTZ19R (lanes 2, 3) and the sequence confirmed. Lane 1; kb ladder.

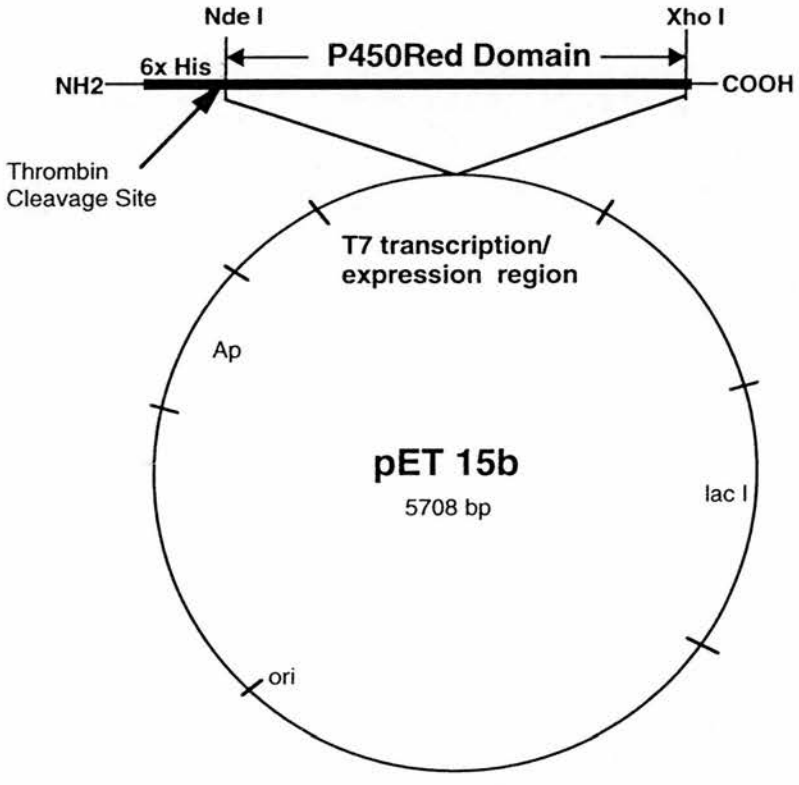



**Figure 4.4**. The expression vector pET15b. The cDNAs encoding the domains shown in Figure 4.2 were cloned into the unique *Nde1*/*Xho1* sites, thus engineering a 6X His-tag and thrombin cleavage site onto the N-terminus of the expressed proteins.

the bacteria. A major reason for the production of inclusion bodies can be due to the strength of the promoter used. The rampant nature of the T7 promoter, found in pET15b, can lead to high production levels of protein resulting in the saturation of the protein folding mechanisms in *E.coli*. This leads to large amounts of unfolded protein within the bacteria which results in the production of inclusion bodies. In Figures 4.5-4.11 it can be observed that for all the proteins expressed some if not all of the protein expressed was insoluble. Indeed, no soluble protein could be produced for the FAD/NADPH(small) or FAD domains (Figure 4.8,4.9). These domains could only be purified under denaturing conditions. In stark contrast, the FMN domain produced large amounts of soluble protein, up to 35 mgs of pure protein from a 1 litre culture (Figure 4.6). The expression and characterisation of the domains shown in Figure 4.2 will be discussed individually in the following sections.

### 4.2.4. Expression, purification and characterisation of the FMN(small) domain of human P450 oxidoreductase.

Figure 4.5 shows the expression and purification of the FMN(small) domain of human P450 reductase. When this 'minimal domain' was expressed the majority of the protein was found to be insoluble. However, some protein was purified from the soluble fraction of the lysed bacteria eluting from the nickel-agarose column at an imidazole concentration of 20 mM. Around 1 mg of FMN(small) protein could be purified from a 1 liter culture of *E.coli* induced with 0.5 mM IPTG for 2 hours. What was evident from looking at the purified protein was that no flavin had been incorporated. There was no evidence for FMN incorporation as judged by the absorption spectrum of the oxidised purified protein. This region of the reductase extends from amino-acids 77-214 and incorporates all the residues proposed to be involved in the binding of FMN as judged from the alignment of this region of P450 reductase with the flavodoxin from *Desulfovibrio vulgaris* (Porter and Kasper, 1986). This result suggests that regions of the reductase outside amino-acids 77-214 are important for binding the FMN moiety and/or correctly folding the functional FMN binding domain of the protein. The predicted molecular weight of the FMN(small) domain is 15.3 kDa. The molecular weight predicted from SDS-PAGE using a 15% gel was 15 kDa.

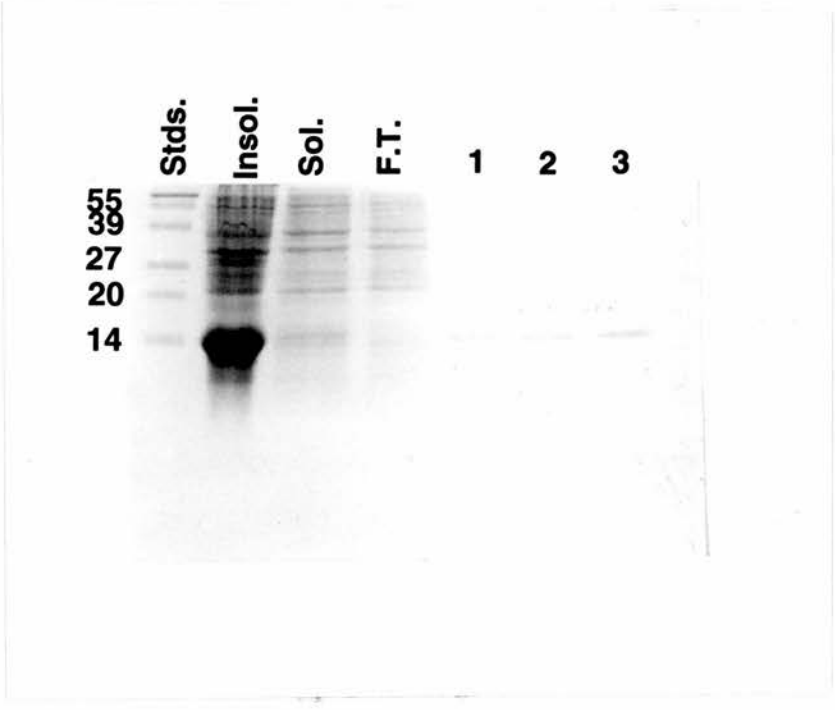



**Figure 4.5. Expression and purification of the FMN (small) domain of P450 reductase.** SDS-PAGE (15%) analysis of the expressed and purified FMN (small) domain. Lanes: Stds, molecular weight standards. Insol., insoluble fraction (10μg); Sol, soluble fraction (10μg); F.T., flow through (10μg). 1-3 Protein eluted with binding buffer + 20mM imidazole.

### 4.2.5. Expression, purification and characterisation of the FMN domain of human P450 oxidoreductase.

Figure 4.6A shows the expression and purification of the FMN domain of P450 reductase. Around 40% of the expressed protein was found to be in the soluble fraction of the lysed bacteria. This domain was found to elute from the nickel-agarose column with an imidazole concentration of 60 mM. From a 1 litre culture of *E.coli*, induced for 2 hours with 0.5mM IPTG, 35 mg of purified FMN domain could be obtained.

Some interesting observations were made when purifying this domain. On preparing the soluble bacterial extract, before loading onto nickel-agarose, it was found to be a very dark grey colour. On addition of the extract onto the nickel-agarose column, the protein that bound to the top of the column appeared very dark blue, almost black in colour. However, on washing the column the colour of the bound protein changed from dark blue to green and then eventually to yellow. When eluted from the column the purified protein appeared yellow. What can initially be inferred from these observations is that incorporation of flavin into the FMN domain had taken place. As regards the colour changes on the column, it can be speculated that the FMN domain was initially isolated from the bacteria in a reduced form. The one electron reduced forms of FMN containing proteins, such as flavodoxins, are known to be air-stable and have high absorbance in the red region of the visible spectrum. This results in the proteins appearing blue when in a one electron reduced state. The absorption spectrum of the oxidised FMN domain is shown in Figure. 4.12A while that of an air-stable reduced form is shown Figure 4.12B. The oxidised spectrum contains absorption maxima at 370nm, 453nm and a broad absorption band between 570nm and 630nm (most probably due to some reduced FMN being present in the sample). This spectrum is virtually identical to that obtained by Kurzban *et al.* (1990) for FAD-depleted P450 reductase and similar to that predicted from a computer model by Oprian and Coon (1982). The spectrum also shows strong similarities to the flavodoxin from *Peptostreptococcus elsdenii* (Iyanagi *et al.*, 1974). In an attempt to chemically reduce the FMN domain an experiment was carried out which involved adding a 10 molar excess of sodium dithionite to the sample. Since it is known that the FMN in native P450 reductase can

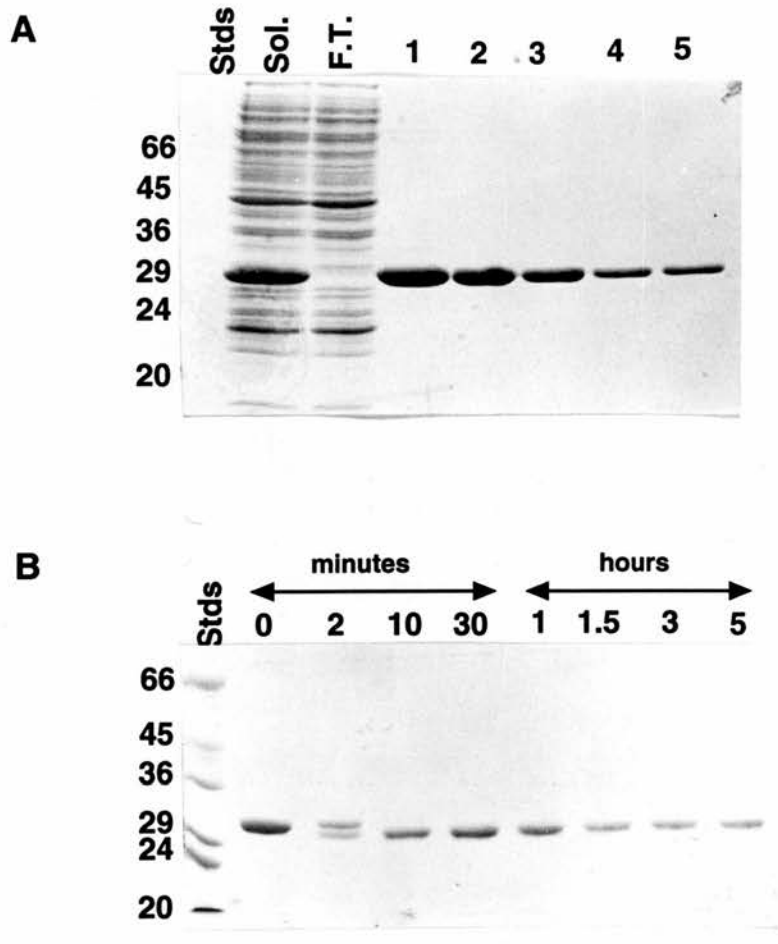



**Figure 4.6. Expression, purification and thrombin cleavage of the FMN domain of P450 reductase. (A)** SDS-PAGE (12%) analysis of the expressed and purified FMN domain. Lanes: Stds, molecular weight standards. Sol, soluble fraction (15μg); F.T., flow through (15μg). 1-4 Protein eluted with binding buffer + 60mM imidazole. **(B)** SDS-PAGE (12%) analysis of the FMN domain after thrombin cleavage. Stds, molecular weight standards. Times of incubation with thrombin are shown.

remain in a one electron reduced state for a long period (up to 48 hours) (Iyanagi and Mason, 1973) it was presumed that a similar state may be achieved for the FMN domain. The two electron reduced FMN in native P450 reductase is unstable and passes an electron onto oxygen (Backes, 1993). This results in the semiquinone form of FMN in P450 reductase being produced. What was anticipated in the experiment described here was that the excess of reducing equivalents would be passed onto molecular oxygen leaving only the one-electron reduced FMN. The result of adding an excess of sodium dithionite to the FMN domain is shown in Figure 4.12B. This spectrum was achieved two hours after the reductant was added and did not change for up to 4 hours. This rate of oxidation, compared to the native reductase, has been observed previously for the FAD-depleted reductase (Kurzban *et al.*, 1990). Compared to the oxidised spectrum (Figure 4.12A vs 4.12B) the air stable reduced spectrum had an increase in absorbance at 585nm with a prominant shoulder at 630nm, while there is a decrease in absorbance at 453nm. An absorbance maximum at 345nm is also observed with a decrease at 370nm. This spectrum is identical to that of the air-stable FAD-depleted reductase produced by photoreduction (Kurzban *et al*, 1990) and similar to that predicted by a computer model (Oprian and Coon, 1982). The spectrum shown in Figure. 4.12B is also similar to the air-stable, one elecron reduced form of the flavodoxin from *Peptostreptococcus elsdenii* (Mayhew and Massey, 1969; Iyanagi and Mason, 1973).

Determination of flavin content revealed that FMN was incorporated in the protein at a ratio of 0.63 mol per mol of protein. This is below the predicted 1:1 ratio and may reflect a loss of flavin during purification. FMN is known to be less tightly bound to the native protein than FAD (Vermillion and Coon 1978b, Kurzban *et al.*, 1990). Another reason may be that for the correct incorporation of FMN into the protein FAD and/or the FAD domain is required. In support of the former argument, it is found that FMN is more easily removed from FAD-depleted reductase (Kurzban and Strobel, 1986, Kurzban *et al.*, 1990).

Since this protein bound FMN it was decided that for further studies the $NH_2$-terminal 6xHis-tag would be removed by thrombin cleavage (Figure 4.6B). The His-tag was easily be removed from this domain in 10 minutes using a ratio of thrombin to domain of 1:5,000. The shift in mobility as judged by SDS-PAGE is clearly shown in Figure 4.6B, giving an estimated size of the FMN domain of 28 kDa. in comparison to the predicted size of 24.2 kDa.

### 4.2.6. Expression, purification and characterisation of the FMN/Anchor domain of human P450 oxidoreductase.

Figure 4.7B shows the expression and purification of the FMN/anchor domain of P450 reductase. The majority of this protein was found to be in the insoluble fraction of the lysed bacteria. This insolubility problem is most probably due to the hydrophobic nature of the $NH_2$-terminus of P450 reductase that is present in exon 2 (amino-acids 1-60). This region contains a long hydrophobic stretch extending from residues 20-44 followed by a cluster of basic residues (amino-acids 45-48), which is characteristic of a simple eukaryotic transmembrane segment (von keijne, 1985). Hydrophobic proteins are known to be problematic when expressed in *E.coli.* (Williams *et al.*, 1982). Despite this, up to 5 mg of the protein from a 1 liter culture could be purified. This domain was eluted from the nickel agarose column by an imidazole concentration of 300 mM.

The yellow appearance of the purified sample suggested flavin incorporation had taken place. The flavin content of the protein was estimated to be 0.67 mol flavin/ mol protein. The oxidised absorption spectrum of the FMN/anchor is shown in Figure 4.13. The most apparent aspect of this spectrum, in comparison to the FMN domain (Figure 4.12A), is the central peak at 420 nm. Apart from this peak the spectrum appears identical to that for the oxidised FMN domain (Figure 4.12A). One explanation for the peak at 420nm may be that there is a heme-contamination from the nickel-agarose purification since free heme would produce a peak in this region of the absorption spectrum. However, after purifying the other soluble FMN domains and FAD/NADPH domain there was no evidence for a similar contamination as judged by the absorption spectrum of the purified proteins (Figures 4.12A and 4.14A). Also, when a control purification was carried out using the soluble extract from non-expressing BL21, no heme was observed to be purified. This peak at 420nm was also unable to be removed by dialysis. Another explanation may be that the contaminant comes from the co-purification of a heme containing protein from the BL21. The gel of the FMN/anchor purification reveals no obvious presence of a contaminating band. However, some minor contaminating bands can be seen in Figure 4.7B when attempts were made on removing the His-tag from the $NH_2$-terminus of the protein. If the contaminant is a cytochrome then it most likely has an extinction

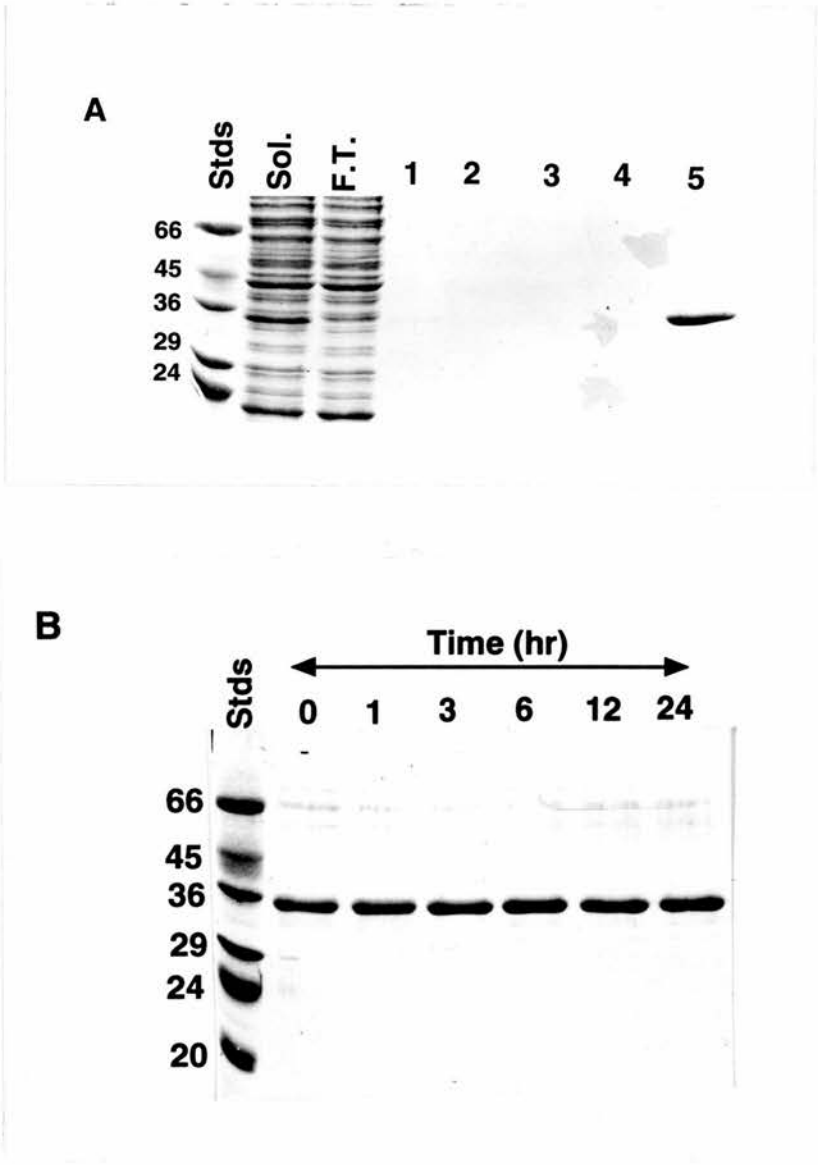



**Figure 4.7. Expression, purification and thrombin cleavage of the FMN/anchor domain of P450 reductase. (A)** SDS-PAGE (12%) analysis of the expressed and purified FMN/anchor domain. Lanes: Stds, molecular weight standards. Sol, soluble fraction (15μg); F.T., flow through (15μg). 1-4 binding buffer + 60mM Imidazole; Lane 5, Binding buffer + 300mM imidazole. **(B)** SDS-PAGE (12%) analysis of the FMN/anchor domain after thrombin cleavage. Stds, molecular weight standards. Times of incubation with thrombin are shown.

coefficient that is ten times that seen for flavins in this range of the visible spectrum. Hence, a cytochrome may represent a contaminant of up 10% in the FMN/anchor preparations. The only difference between this domain and the FMN domain is the first 60 amino-acids, which are predominatly hydrophobic. This region of the reductase has been implicated in membrane binding and P450 interactions (Black *et al.*, 1979). It may be speculated that this region of the reductase is interacting with a bacterial cytochrome. If an interaction with a bacterial cytochrome is taking place then it is probably hydrophobic in nature since the purification was carried out in high salt concentration (500 mM NaCl). It is interesting to note that studies on the bacterial expression of cytochrome P450 CYP17A (Barnes *et al.*, 1991) found that P450 monooxygenase activity could be reconstituted from bacterial membranes without the exogenous addition or co-expression of P450 reductase. Recent, unpublished, work from the laboratory of Waterman has found that the heterologously expressed cytochrome P450 CYP17A was interacting with the *E.coli* flavodoxin (Waterman, personal communication). Since this domain was able to bind flavin, it was decided to be used for further studies despite the presence of a contaminant. The removal of the 6xHis-tag was attempted using thrombin. Unfortunately, as shown in Figure. 4.7B, the removal of this linker from the N-terminus of the protein was unsuccessful, even at a ratio of thrombin:protein of 1:1000. This is in contrast to the FMN domain (Figure 4.6B). Accessibility to the thrombin cleavage site by the protease is most probably the determining factor in this instance.
Determination of flavin content revealed that FMN was incorporated in the protein at a ratio of 0.67 mol per mol protein.

### 4.2.7. Expression, purification and characterisation of the FAD domain of human P450 oxidoreductase.

Expression of the FAD domain of P450 reductase (Figure 4.8) resulted in the production of only insoluble protein. No soluble protein could be purified from the soluble fraction of the *E.coli* (Figure 4.8A). This protein was purified from inclusion bodies under denaturing conditions (Figure 4.8B). The protein was found to elute from the nickel-agarose at an imidazole concentration of 20 mM in the presence of 6 M urea. Initial attempts at refolding this domain were unsuccessful. Further refolding experiments of this protein to a soluble form without the presence of urea were not performed. The denatured FAD

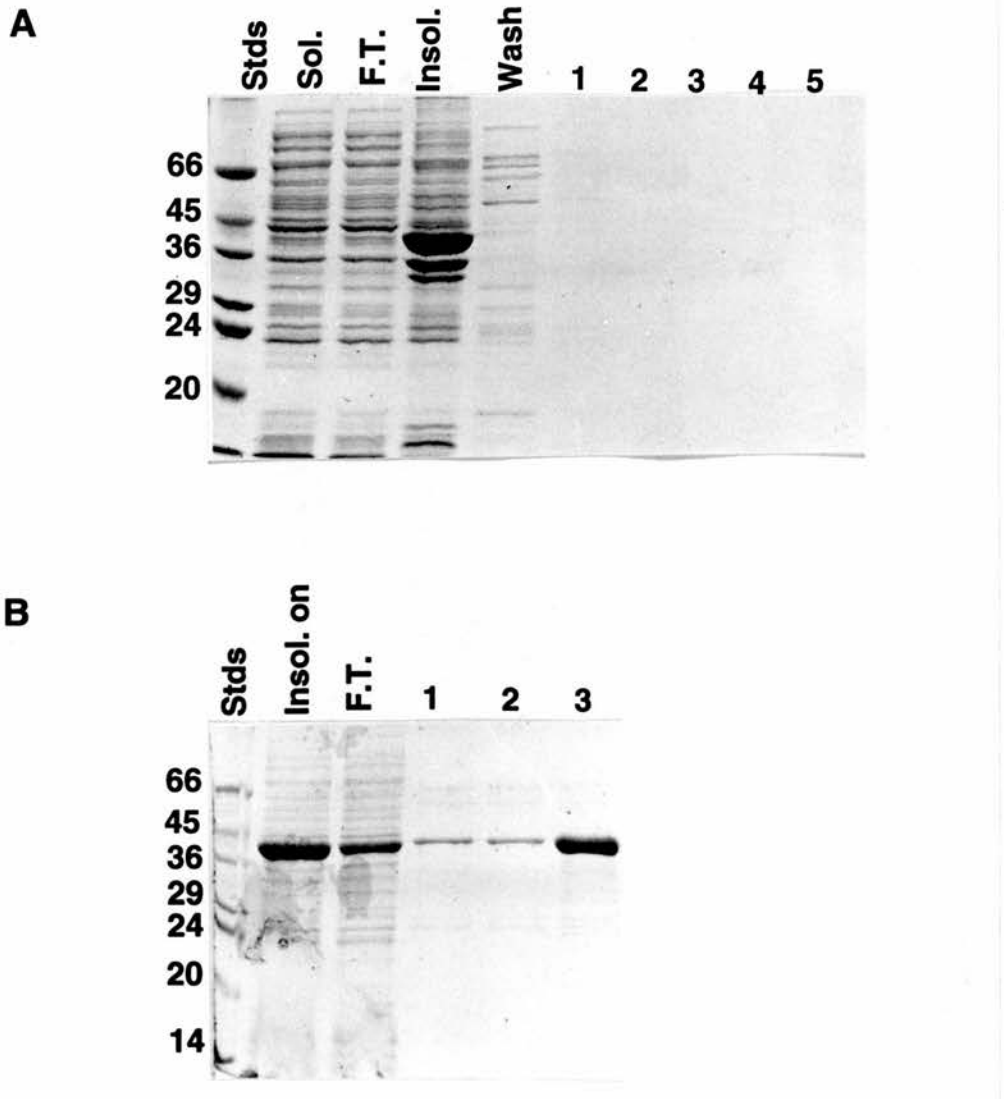



**Figure 4.8. Expression and purification of the FAD domain of P450 reductase.** **(A)** SDS-PAGE (12%) analysis of the expressed and purified FAD domain under non-denaturing conditions. Lanes: Stds, molecular weight standards. sol., Soluble fraction loaded onto nickel-agarose; F.T. flow through; insol., insoluble fraction; wash, wash with binding buffer; 1-3 protein eluted with binding buffer +60mM imidazole; 4-5 protein eluted with binding buffer + 1M imidazole. **(B)** SDS-PAGE (12%) analysis of the expressed and purified FAD domain under denaturing conditions.Insol. on, insoluble fraction loaded onto Nickel-agarose; F.T., flow through; 1,2 Protein eluted with binding buffer + 20mM imidazole + 6M urea; 3, protein eluted with Binding buffer + 300mM imidazole + 6M urea.

domain had an apparent molecular weight of 42 kDa., as judged by SDS-PAGE and a predicted molecular weight of 41.0 kDa.

This domain contains the majority of the residues proposed to be involved in the binding of FAD as judged by the sequence alignment with spinach FNR whose crystal structure is known (Karplus *et al.*, 1991). However, at the C-terminus of FNR, amino acids Tyr 314 and Glu 312 are involved in shielding the face of the flavin and making some contact with the dimethylbenzyl ring respectively. These amino-acids align with Trp 676 and Glu 674 of the human reductase and hence will not be present in the construct described here. Also, this construct did not contain a significant portion of the NADPH binding domain as judged from the alignment with spinach FNR. When describing the structure of spinach FNR, the authors stated that due to the large contact area between the FAD and NADPH domains it would not be possible to functionally separate these two domains (Karplus *et al.*, 1991). The work presented here on the bacterial expression of this region of P450 reductase suggests that the FAD/NADPH binding region of P450 reductase cannot be separated into distinct functional units.

### 4.2.8. Expression, purification and characterisation of the FAD/NADPH(small) domain of human P450 oxidoreductase.

Figure 4.9 shows the expression and purification of the FAD/NADPH(small) domain of P450 reductase. This 'minimal' FAD/NADPH domain contains all the critical amino-acid residues for FAD and NADPH binding. These critical residues were derived from alignment of P450 reductase with spinach FNR (Karplus *et al.*, 1991). However, this protein could not be obtained in a soluble form and was purified under denaturing conditions (Figure 4.9A, 4.9B). The protein eluted from the nickel-agarose column with 20 mM imidazole in the presence of 6 M urea. However, a large proportion of the protein passed through the column without binding. The molecular weight as judged by SDS-PAGE on a 12% gel was 31 kDa in comparison to the predicted weight of 30.2 kDa. Similar to the FAD domain, initial attempts at refolding this protein proved fruitless and no further trials at renaturation were carried out. Potential reasons for the inability to obtain this protein in a soluble and functional form will be discussed at the end of the next section (4.2.9) which discusses the expression, purification and characterisation of a larger FAD/NADPH domain.

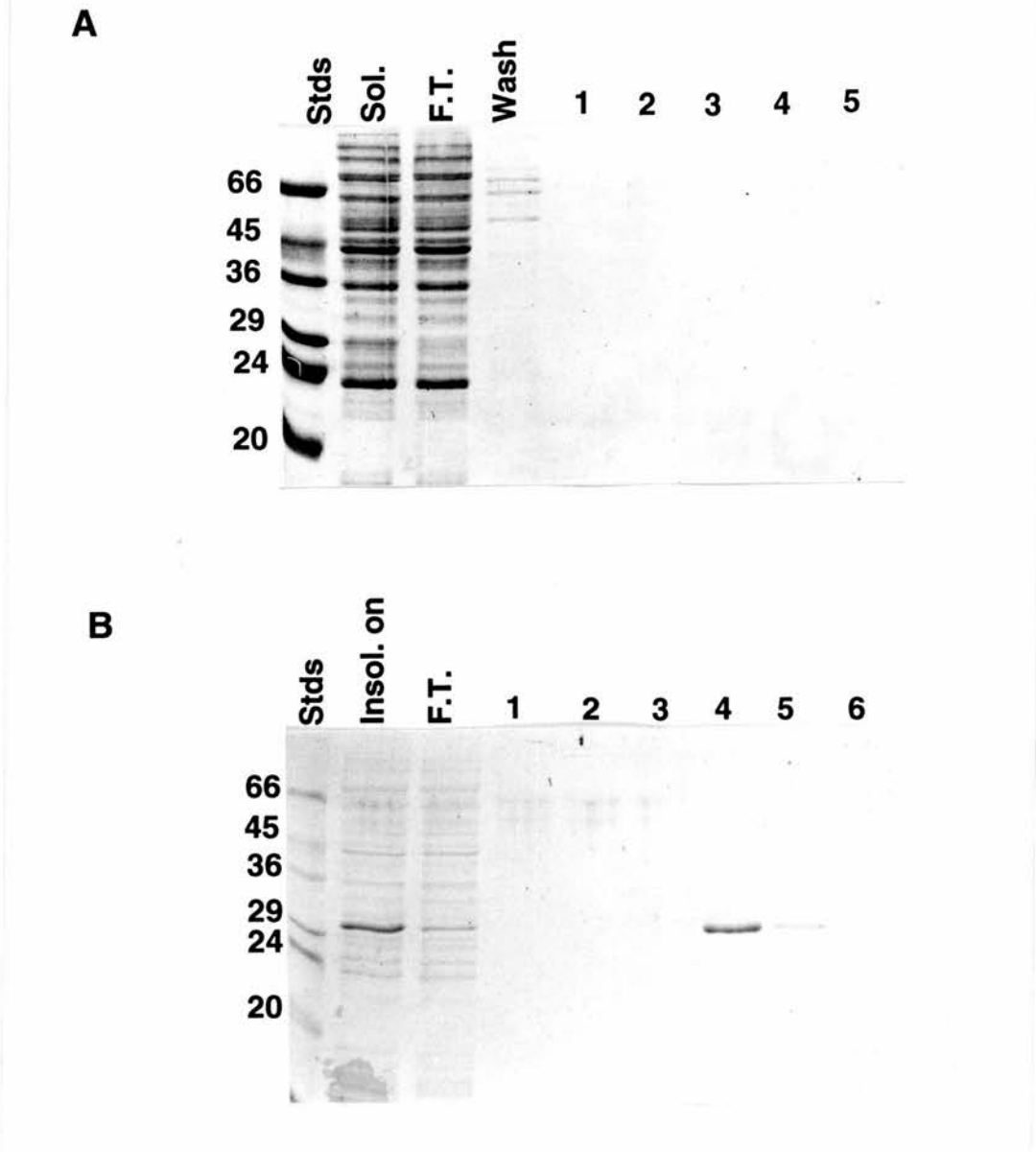



**Figure 4.9. Expression and purification of the FAD/NADPH (small) domain of P450 reductase.** **(A)** SDS-PAGE (12%) analysis of the expressed and purified FAD/NADPH domain under non-denaturing conditions. Lanes: Stds, molecular weight standards. sol., Soluble fraction loaded onto nickel-agarose; F.T. flow through; wash, wash with binding buffer; 1-3 protein eluted with binding buffer +60mM imidazole; 4-5 protein eluted with binding buffer + 1M imidazole. **(B)** SDS-PAGE (12%) analysis of the expressed and purified FAD/NADPH (small) domain under denaturing conditions. Insol. on, insoluble fraction loaded onto Nickel-agarose; F.T., flow through; 1,2,3 Protein eluted with binding buffer + 20mM imidazole + 6M urea; 4,5,6 protein eluted with Binding buffer + 300mM imidazole + 6M urea.

### 4.2.9. Expression, purification and characterisation of the FAD/NADPH domain of human P450 oxidoreductase.

Figure 4.10A shows the expression and purfication of the FAD/NADPH domain of human P450 reductase. As can be seen from this Figure, the majority of the protein expressed ended up in the insoluble fraction of the bacteria. However, up to 3.5 mg of the FAD/NADPH domain could be purified from the soluble bacterial fraction obtained from a 1 litre culture of *E.coli* induced with 0.5 mM IPTG for 2 hours. This protein eluted from the nickel-agarose column with an imidazole concentration of 60 mM. The estimated molecular weight of the protein as judged by SDS-PAGE, after thrombin cleavage, was 50 kDa in comparison to the predicted size of 49.7 kDa.
Similar to the FMN and FMN/anchor domains, the purified protein appeared yellow suggesting the incorporation of flavin into this domain. The absorption spectrum of the FAD/NADPH domain is shown in Figure 4.14A. This spectrum shows absorption maxima at 382nm and 454nm. The spectrum is virtually identical to that produced by FMN-depleted native P450 reductase (Vermillion and Coon, 1978b; Iyanagi *et al.*, 1981; Vermillion and Coon; 1981; Kurzban and Strobel; 1986). However, the FAD/NADPH domain spectrum shows considerably more definition to the spectrum of the FMN depleted reductase produced by site-directed mutagenesis (Shen *et al.*, 1989). Mutagenesis may not only perturb the local environment of the protein but may also have a global effect on the structure of the whole protein. In the case of P450 reductase, mutagenising tyrosine residues involved in the binding of FMN (Shen *et al.*, 1989) may have perturbed the overall structure of the protein and affected the incorporation of FAD into the mutated protein and/or affected the orientation of the FAD in its binding site. These maybe the reasons for the poorly defined spectrum obtained by Shen *et al.*, (1989) and also for the reduced incorporation of FAD found in their mutated protein. Similar to the FMN and FMN/anchor domains the FAD/NADPH domain contained less than the predicted amount of flavin. The ratio of FAD:protein was calculated as 0.70:1. This reduced flavin content may reflect a loss of flavin during purification and/or a requirement for the FMN domain of the protein for correct incorporation/stabilisation of the FAD moiety. In the early work that was performed on removing the more labile FMN moiety from native P450 reductase it was observed that the FAD was less tightly bound in

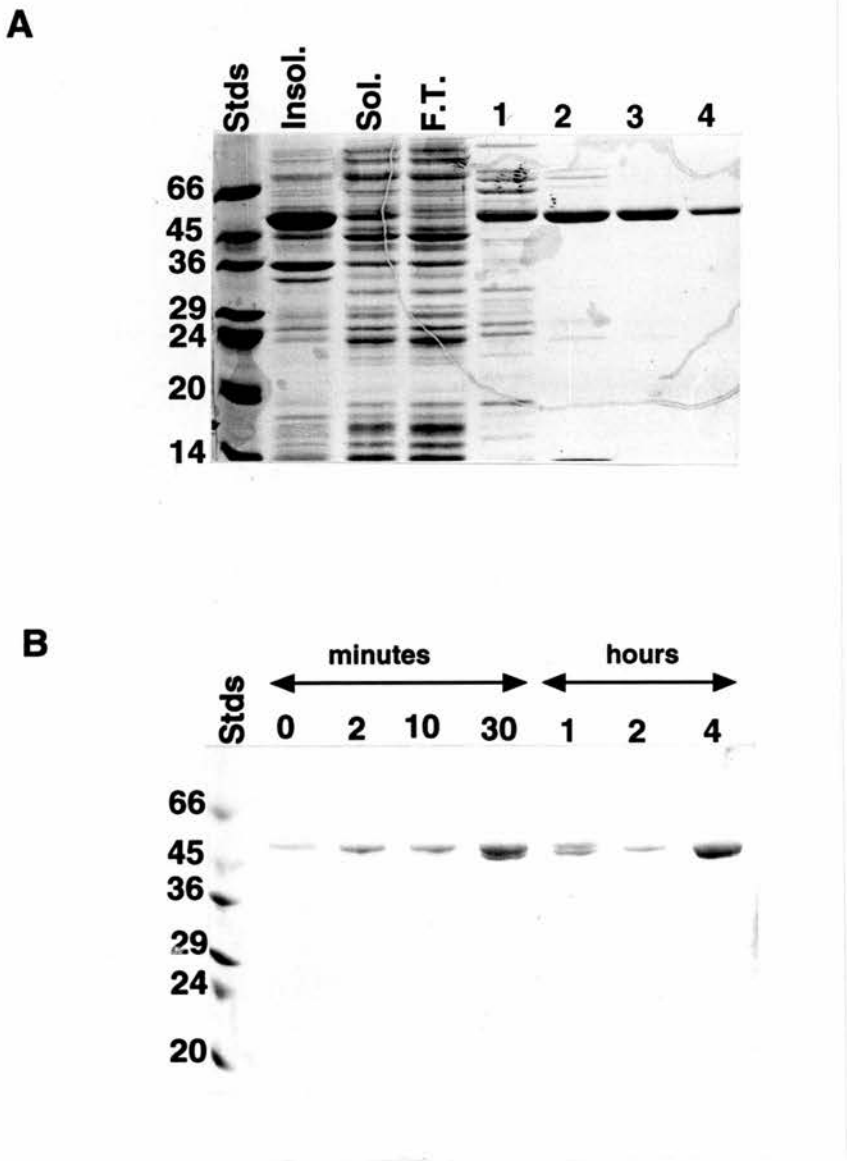



**Figure 4.10. Expression, purification and thrombin cleavage of the FAD/NADPH domain of P450 reductase. (A)** SDS-PAGE (12%) analysis of the expressed and purified FAD/NADPH domain. Lanes: Stds; Molecular weight standards; Insol, insoluble fraction (15µg); sol, soluble fraction loaded onto the Nickel-agarose column (15µg); F.T. Flow through (15µg); 1-4, Fractions eluted with binding buffer + 60mM imidazole. **(B)** SDS-PAGE (12%) analysis of thrombin cleaved FAD/NADPH domain. Stds., molecular weight standards; Times of incubation with thrombin are shown.

the FMN-depleted reductase (Vermillion and Coon, 1978b; Iyanagi *et al.*, 1981; Vermillion and Coon; 1981; Kurzban and Strobel; 1986). These workers have suggested that some cooperativity exists between the flavins and that both require one another for stabilisation. Bonants *et al.* (1990) found alterations in the FAD P31 NMR resonance upon removal of FMN suggesting altered binding of FAD in the FMN-depleted reductase. However, what is clear from the results shown in this study is that incorporation of each flavin into its respective domain is not entirely dependent on the other flavin and/or domain.

Functional analysis of electron transfer reactions within P450 reductase have concluded that electrons flow from NADPH through the low potential flavin (FAD) to the high potential flavin (FMN) and then onto the cytochromes P450 (reviewed by Backes, 1993). Reduction of the FAD/NADPH domain with a 10 fold molar excess of NADPH, under aerobic conditions, resulted in alterations in the oxidised absorption spectrum (Figure 4.14A) as shown in Figure 4.14B. A decrease in the absorbance maximum at 454nm can be observed with the production of an absorbance peak at 592nm after 10 minutes. This is indicative of the production of an FAD semiquinone moiety (Vermillion and Coon, 1978b; Vermillion *et al.*, 1982)). Unlike the FMN semiquinone, which was stable in air for several hours (Figure 4.12B) this species was not stable and after 25 minutes under aerobic conditions the spectrum had reverted back to that of the oxidised FAD/NADPH domain (Figure 4.14B). This data is consistent with results obtained for the FMN-depleted reductase (Vermillion and Coon, 1978b; Iyanagi *et al.*, 1981; Vermillion and Coon; 1981; Kurzban and Strobel; 1986). These workers observed that when the FMN-depleted reductase was reduced with NADPH, in air, the reduced FAD quickly reoxidised.

Since this 'functional' domain was to be used for further studies attempts were made at the removal of this 6xHis-tag from the N-terminus of the recombinant protein. Figure 4.10B shows that the tag could be efficiently removed from the $NH_2$-terminus of the protein using a ratio of thrombin:domain of 1:5000. 100% cleavage was found to occur after 4 hours. The shift in mobility as judged by SDS-PAGE using a 12% gel can be seen.

The ability of this domain to reduce a variety of one electron acceptors, and cytochrome P450, is shown in Table 4.1. The data is expressed as mmol of substrate reduced per/ minute/ mg of protein. This data indicates that the FAD/NADPH domain is catalytically active towards a variety of substrates.

Table 4.1: Specific Activity of P450 reductase and domains.

| | | Activity* | | |
|---|---|---|---|---|
| Substrate | P450 red. | FAD/NADPH | FAD/NADPH +FMN | FMN |
| Ferricyanide | 33.1 | 17.9 (54.1%) | 16.1 (48.6%) | N.D. |
| DCPIP | 11.2 | 0.456 (4.1%) | 0.705 (6.3%) | N.D. |
| Menadione | 7.67 | 0.145 (1.9%) | 0.134 (1.7%) | N.D. |
| 3-AcPy-ADP | 1.14 | 0.228 (20%) | 0.207 (18%) | N.D. |
| CYP1A1 | 6.34 | 0.001 (0.016%) | 0.121 (1.9%) | N.D. |
| Cytochrome c | 23.5 | 0.025 (0.11%) | 0.410 (1.7%) | N.D. |

Specific activities are expressed as µmols substrate reduced/minute/mg protein except for menadione where the oxidation of NADPH was followed. CYP1A1 activity was determined by measuring the oxidative O-deethylation of ethoxyresorufin and is expressed as nmol resorufin produced/min/ nmol P450. All assays were carried out in triplicate with SEM< 5%. N.D., not detectable

However, the ability to reduce these compounds is diminished by varying degrees compared to native P450 reductase. Of particular note is the poor activity that the FAD/NADPH domain exhibits towards CYP1A1 and cytochrome c. The data here is found to be very similar to that obtained for the FMN-depleted reductase (Vermillion and Coon, 1978b) except for the reduction of cytochrome P450 and cytochrome c. This is most probably due to the removal of the FMN domain in the construct described here. Regions within the FMN domain have been shown to interact directly with rat CYP2B1 (Nelson and Strobel, 1991) and cytochrome c (Nisimoto, 1986). In FMN depleted reductase not all the FMN is able to be removed and so some functional protein is still present as well as the substrate interaction site(s). In comparison, the FAD/NADPH domain described here does not contain the FMN binding region thus removing the P450 and cytochrome c interaction site along with the FMN prosthetic group.

In comparison to the FAD/NADPH(small) domain (4.2.8), the protein described in this section had an additional 172 amino acids at the $NH_2$-terminus (Exons 8-11). Since no functional protein could be obtained for the FAD/NADPH(small) domain it can be concluded that exons 8-11 or regions within this part of the protein are important for the overall folding of the protein and/or for the incorporation of FAD. In the early alignments of the rat P450 reductase protein with a variety of flavoproteins (Porter and Kasper, 1986) it was found that amino-acids 266-325 (amino- acids 267-326 of the human reductase) showed weak homology with the amino terminus of spinach FNR and cytochrome b5 reductase (homology over this region can be seen in Figure 4.1C). Porter and Kasper (1986) suggested that these amino-acids might be important for interaction with the pyrophosphoryl region of the FAD. However, after the structure of spinach FNR became available (Karplus *et al.*, 1991) it became evident that this region of FNR was not important for interaction with the pyrophosphoryl group. This may not preclude amino-acids within this region of P450 reductase from interacting with FAD. Removal of this region from the FAD/NADPH domain, producing the FAD/NADPH(small) domain, appears to prevent formation of a protein able to bind FAD. Interestingly, following this stretch of weak homology with cytochrome b5 reductase and FNR (amino-acids 266-325) there is a region of the protein termed 'the insertion' (Porter and Kasper) which appears not to align with any other proteins apart from members of the FMN/FAD containing family *i.e.* P450 reductase, sulfite reductase and nitric oxide

synthase. Porter and Kasper (1986) proposed that this insertion is necessary for the proper positioning of the FMN and FAD moieties and for efficient transfer. This insertion would not be required in FMN only and FAD only containing flavoproteins. Therefore, this region may be required for helping to maintain the FAD in its binding site. It does not appear to be of major importance in FMN binding or orientation as shown in Figure 4.12A,B but may be another factor for the less than stoichiometric amounts of FMN found in the FMN and FMN/anchor domain.

### 4.2.10. Expression, purification and characterisation of the FMN/FAD domain of human P450 oxidoreductase.

The results of the expression and purification of the FMN/FAD domain are shown in Figures 4.11A,B. A very small percentage of this protein was found to be soluble. However, on purification this protein was extremely susceptible to proteolysis despite the presence of a cocktail of protease inhibitors in the purification (Figure 4.11A). The purification shown in Figure 4.11B was carried out under denaturing conditions. The estimated molecular weight of this protein was 69 kDa. as judged by SDS-PAGE using a 12% gel, in comparison to the predicted molecular weight of 61.3 kDa.
Similar to the FAD domain described in section 4.2.7, this region of P450 reductase did not contain the last 75 amino acids, *i.e.* exons 15 and 16. Therefore, as described in section 4.2.7, it does not fully qualify as the FAD domain. The protein produced will probably contain both structured and unstructured regions from the FMN binding region and the partial FAD binding region respectively. This will most probably lead to the production of an unstable protein which may be the reason for its susceptibility to proteolysis. It appeared form the colour of the degraded sample that some flavin was present but this was not formally identified.

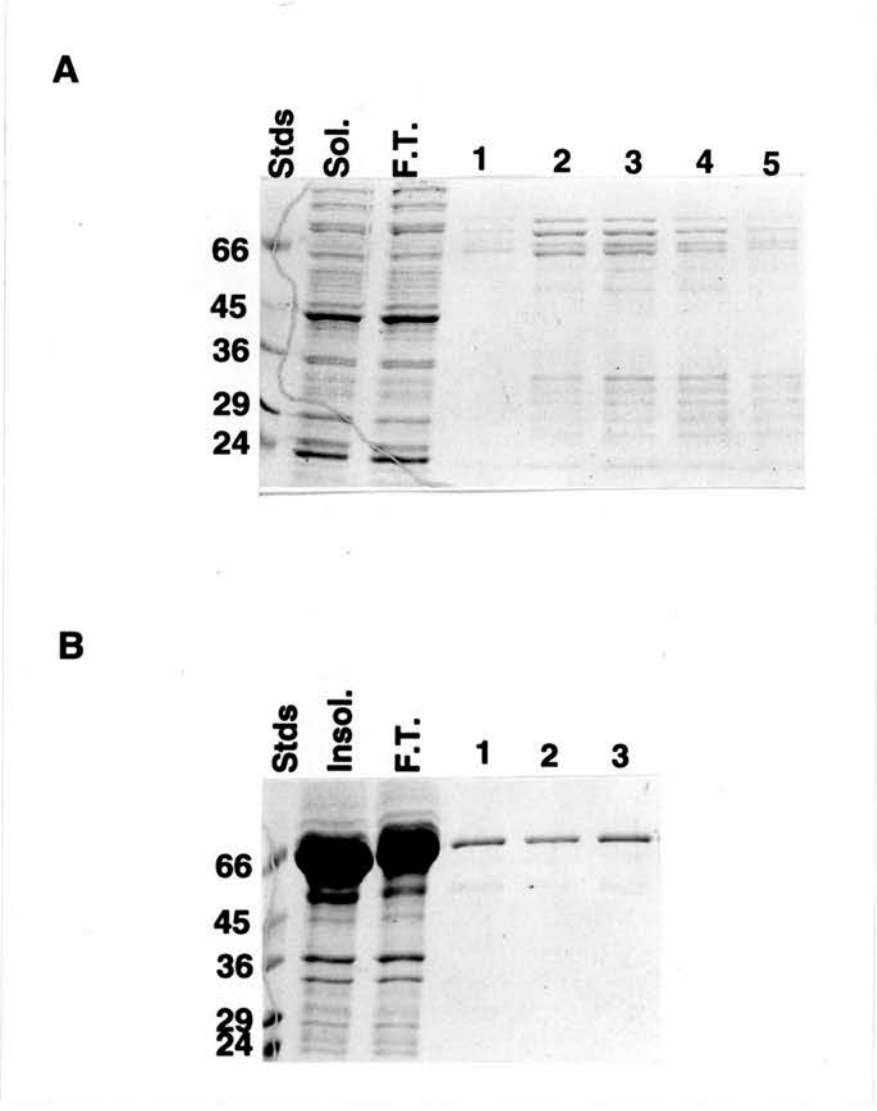



**Figure 4.11. Expression and purification of the FMN/FAD domain.** **(A)** SDS-PAGE (12%) analysis of the FMN/FAD domain purified from the soluble bacterial extract. Lanes: Stds, Molecular weight standards; sol., soluble fraction (10μg) loaded onto Nickel-agarose; F.T. Flow through; 1,2 fractions obtained from elution with binding buffer + 60mM imidazole. **(B)** SDS-PAGE analysis of the FMN/FAD domain purified under denaturing conditions. Lanes: Stds, molecular weight standards; Insol, insoluble fraction loaded onto Nickel-agarose; F.T. flow through; 1-3, fractions eluted with binding buffer + 20mM imidazole + 6M urea.

## 4.3 Reconstitution studies using the bacterially expressed 'functional' domains of human NADPH cytochrome P450 oxidoreductase.

### 4.3.1 Reconstitution of human NADPH cytochrome P450 oxidoreductase spectra using the FMN and FAD/NADPH domains.

The spectra shown in Figure 4.12 and Figure 4.14 showed that the purified FMN and FAD/NADPH domains could bind their respective flavins independently. In order to investigate whether the FAD/NADPH domain could transfer electrons from NADPH via FAD to the FMN domain, in a manner similar to native P450 reductase, the two domains were mixed together (Figures 4.15A,B). In both native and trypsin treated P450 reductase electrons are transferred from NADPH through FAD and onto FMN (Vermillion and Coon, 1978b; Iyanagi *et al.*, 1981; Vermillion *et al.*, 1982; Kurzban and Strobel, 1986, reviewed in Backes, 1993). When the reductase is reduced under aerobic conditions with an excess of NADPH, an air stable semiquinone species is produced slowly. This semiquinone species has been identified as the one-electron reduced FMN reductase. (Iyanagi and Mason, 1973; Iyanagi *et al.*, 1974; Iyanagi *et al.*, 1978; Vermillion and Coon, 1978a; Yasukochi *et al.*, 1979). A U.V.-visible spectrum characteristic air-stable semiquinone species for human P450 reductase is shown in Figure 4.15A. An increase in absorbance at 585nm with a shoulder at 630nm and a decrease in absorbance at 455nm can be observed. This species is stable for between 24 and 48 hours when left open to air (Horecker, 1950; Masters and Kamin, 1965; Iyanagi and Mason, 1973; Iyanagi *et al.*, 1974; Iyanagi *et al.*, 1978; Vermillion and Coon, 1978a; Yasukochi *et al.*, 1979). If electrons could be transferred from NADPH via the independent FAD/NADPH domain onto the FMN domain then a spectrum similar to Figure 4.15A should be produced indicating FMN semiquinone formation. This semiquinone would be predicted to be air stable. Fig. 4.15B shows the ability of equimolar amounts of the FMN and FAD/NADPH domains to produce a spectrum similar to that of native P450 reductase. When reduced by NADPH the two independent domains do indeed produce a spectrum indicative of an air-stable semiquione species. A decrease in absorbance at 455nm can be observed with a concomitant increase in absorbance at 585nm and a shoulder at 630nm. This however was not as stable as that seen for the reductase purified from

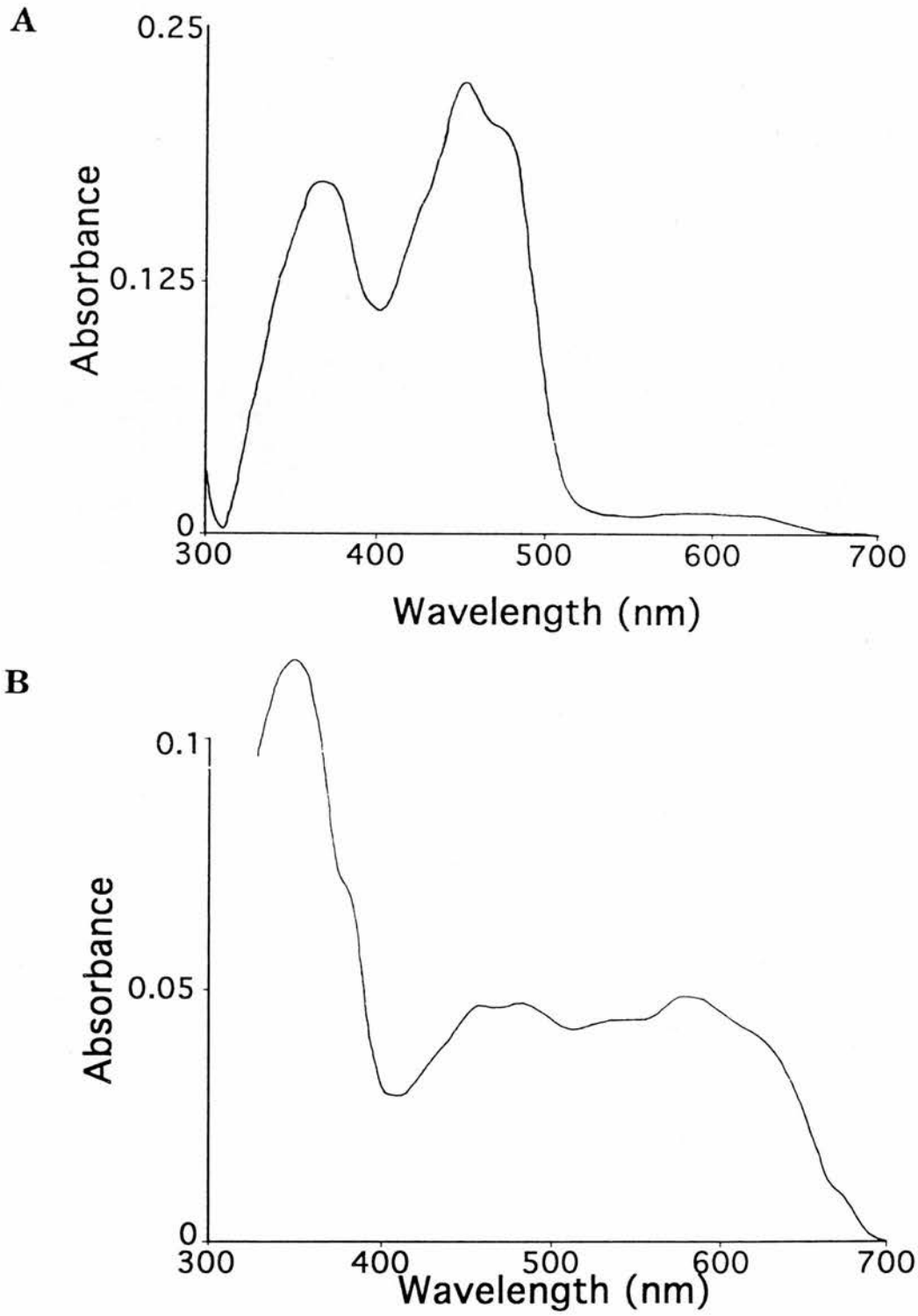



**Figure 4.12 Absorption spectra of the FMN domain of P450 reductase.** **(A)** The oxidised FMN domain, 55μM. **(B)** The air-stable reduced form of the FMN domain (30μM), 2 hours after reduction with an excess of sodium dithionite.

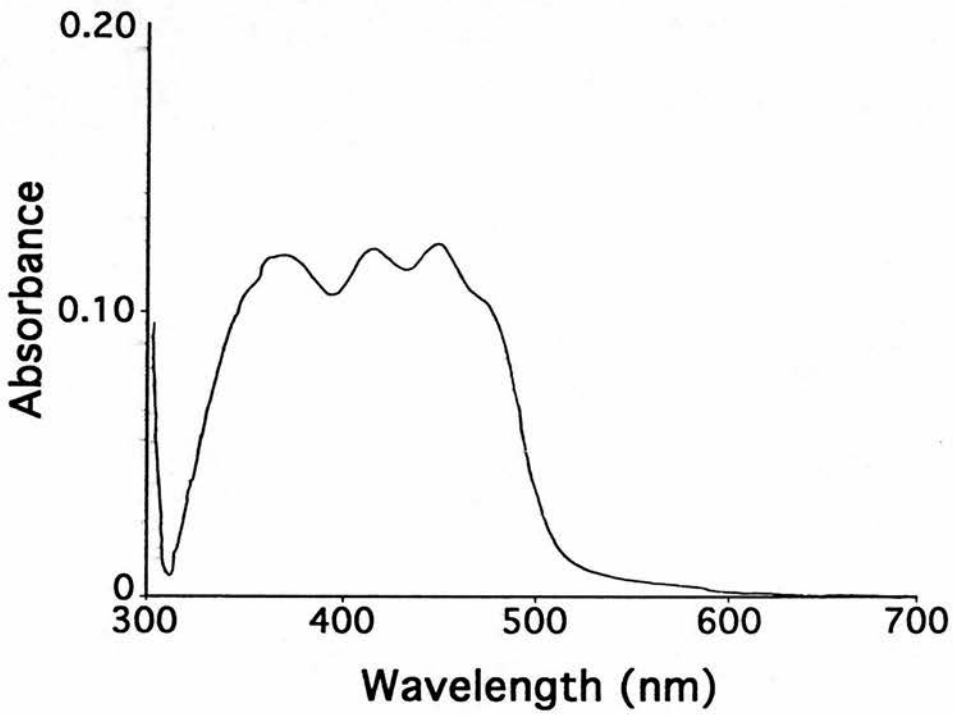



**Figure 4.13** Absorption spectrum of the FMN/anchor domain (oxidised) of P450 reductase (25μM).

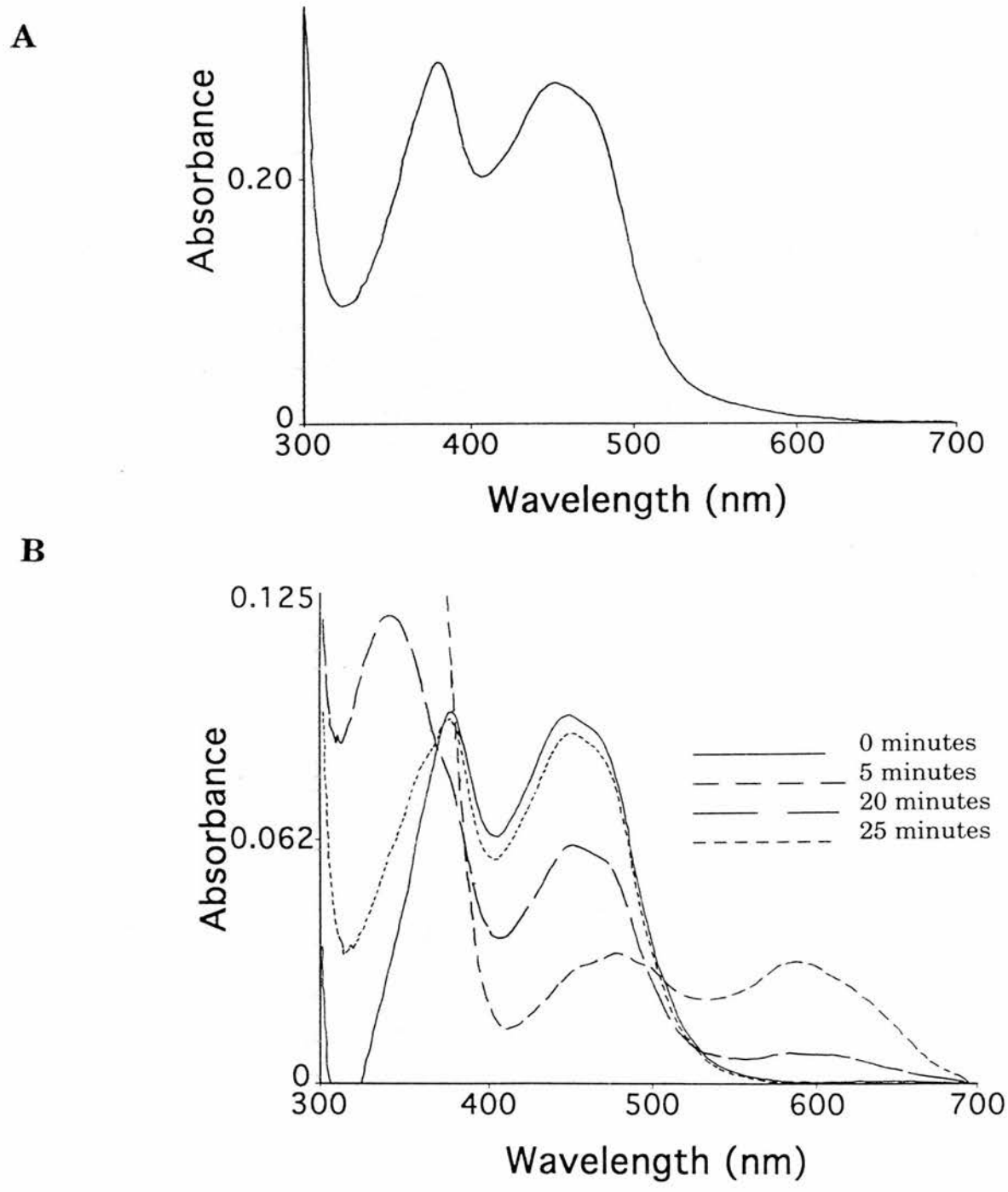



**Figure 4.14 Absorption spectra of the FAD/NADPH domain of P450 reductase. (A)** Oxidised FAD/NADPH domain (50μM). **(B)** FAD/NADPH domain (20μM) after reduction with 200μM NADPH under aerobic conditions. Times after reduction are shown on the spectrum.

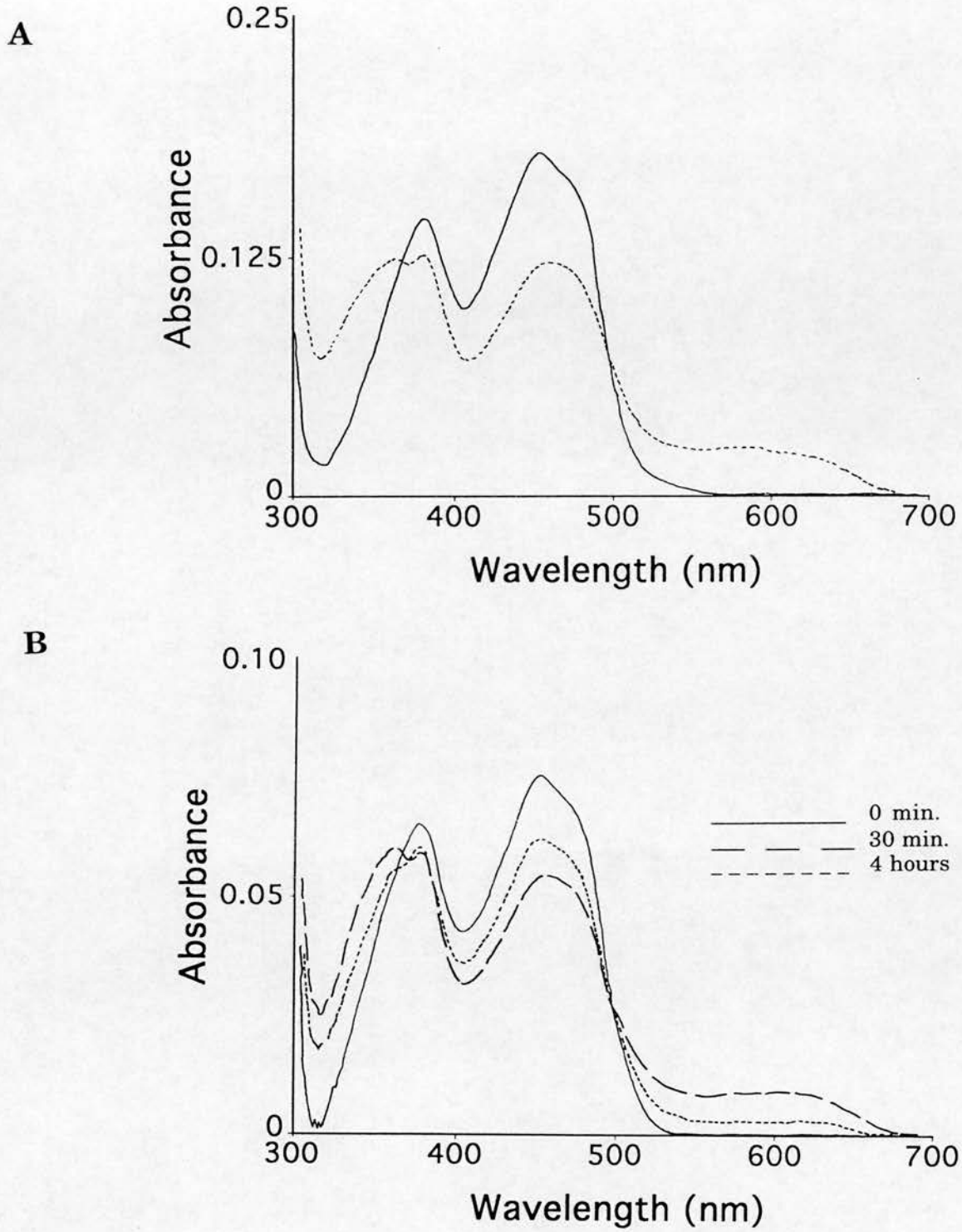



**Figure 4.15. Absorption spectra of native P450 reductase and the mixed FMN and FAD/NADPH domains.** **(A)** Absorption spectra of oxidised native P450 reductase (10μM) and the air stable semiquinone form, 18 hours after reduction with 200μM NADPH. **(B)** Absorption spectra obtained on mixing the FMN (7.5μM) and FAD/NADPH (7.5μM) domains and after reduction with NADPH (200μM). Times after reduction with NADPH are shown on the spectrum.

human liver microsomes (Fig. 4.15A). The reconstituted domains slowly reoxidised after a period of 4 hours in comparison to the purified reductase which maintained its air-stable species for up to 24 hours before reoxidising. This faster reoxidation of the semiquinone species seen for the two mixed domains compared to native P450 reductase maybe due to stability of the FMN semiquinone that the intact protein can provide. Indeed, the air-stable semiquinone of the FMN domain (Figure 4.12B) was stable for a similar time to the air-stable form produced by the mixed domains before slowly re-oxidising.

### 4.3.2 Interaction between the FMN and FAD/NADPH domains.

In view of the observation that electrons could be transferred from NADPH via the FAD/NADPH domain onto FMN it was decided to further examine the interaction between the two domains. The His-tagged FMN domain was mixed with nickel-agarose and a His-tagged-FMN domain nickel-agarose column produced. To this column the FAD/NADPH domain (without the His-tag) was applied. This was carried out in low ionic strength buffer (10mM potassium phosphate, pH7.7) as shown in Figure 4.16. The FAD/NADPH domain only bound to the His-tagged-FMN domain column and not the nickel-agarose alone. The FAD/NADPH domain could be dissociated/eluted from the column by increasing the ionic strength of the buffer (Figure 4.16). This result indicates that ionic interactions are important for the domain interaction to occur.

### 4.3.3. Interaction of cytochrome c with the FMN domain of human NADPH cytochrome P450 oxidoreductase.

The ability of cytochrome c, a well characterised one electron acceptor of P450 reductase, to interact with either the FMN or FAD/NADPH domain was looked at using His-tagged domains bound to nickel agarose columns. This experiment was carried out in a manner similar to that described in 4.3.2. When cytochrome c was applied to the column containing the immobilised FMN domain, in low ionic strength (1mM potassium phosphate, pH 7.7), the protein was retained on the column (Figure 4.17). The cytochrome c could be eluted by increasing the ionic strength of the buffer. In a similar experiment with the His-tagged FAD/NADPH protein immobilised on a nickel agarose

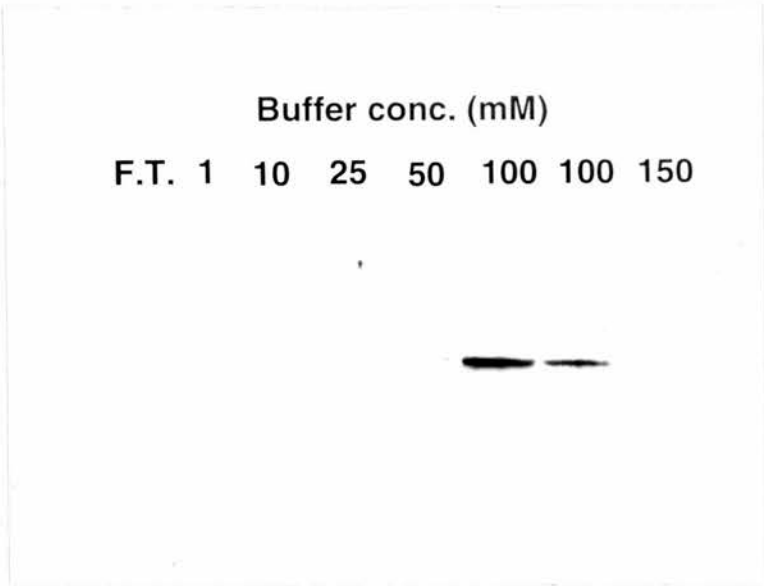



**Figure 4.16. Interaction of the FMN and FAD/NADPH domains**. 2 mg of His-tagged FMN domain was bound to a 1ml nickel-agarose column and the FAD/NADPH domain (0.1mg/ml) applied in 10mM potassium phosphate buffer, pH 7.7. This resulted in the binding of the latter domain. The ionic strength of the buffer passed through the column was increased in a step-wise fashion and the resulting eluates analysed by SDS-PAGE followed by Western blotting using an antibody to full length reductase.

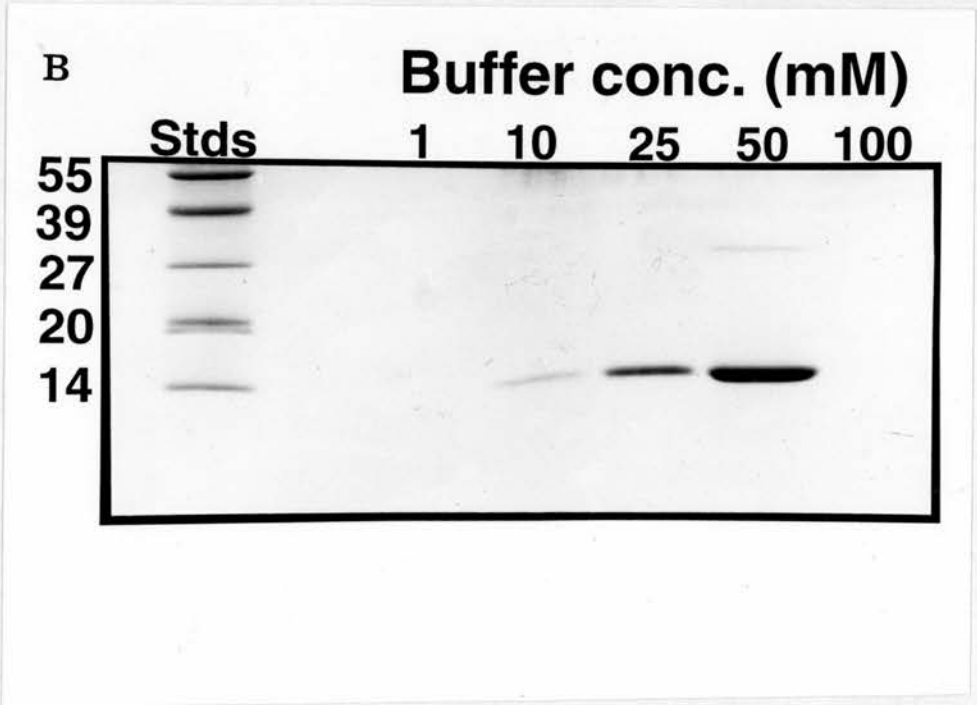



**Figure 4.17 Interaction of the FMN domain with cytochrome c.** 2mg of His-tagged FMN-domain was bound to a 1ml nickel-agarose column and cytochrome c (0.5 mg/ml) applied in 1mM potassium phosphate buffer, pH7.7. This resulted in the cytochrome c binding. The ionic strength of the buffer was then increased in a stepwise manner and eluted fractions analysed by SDS-PAGE (15% gel). F.T., flow through.

column it was found that cytchrome c did not bind. The direct involvement of the FMN domain with cytochrome c binding is in agreement with the previous study of Nisimoto (1986) who was able to cross-link P450 reductase with cytochrome c in low ionic strength buffer. His work indicated that some of the amino-acids in the region 200-220 of the rat P450 reductase are involved in the interaction with cytochrome c. These residues are located in the FMN domain. Ionic strength effects on cytochrome c reductase activity were observed in the early studies of the purified reductase (Williams and Kamin, 1965). These studies showed that by increasing the ionic strength the rate of cytochrome c reduction increased. The standard assay for cytochrome c reduction is carried out in 300mM potassium phosphate buffer, pH7.7. The data in Figure 4.17 and that published previously (Nisimoto, 1986) shows that in low ionic strength buffer the FMN domain reductase can bind with cytochrome c. However, in low ionic strength buffer cytochrome c reductase activity is low. These observations warrent further investigation.

### 4.3.5. Reconstitution of cytochrome c and cytochrome P450 reductase activity using the domains of human NADPH cytochrome P450 oxidoreductase.

The data so far presented in this chapter shows that the recombinant FMN, FMN/anchor and FAD/NADPH domains folded correctly and that an interaction could take place between the FMN and FAD/NADPH domains. It was also observed that the FMN domain could form a complex with cytochrome c. A variety of experiments were carried out to establish whether the domains could couple to form a catalytically competent unit.

Cytochrome c reductase activity could be reconstituted when a combination of the FMN and FAD/NADPH domains was used (Figure 4.18A). The ability to form an active complex was time dependent and had an estimated t1/2 of 3 hours. The final activity of the reconstituted domains was found to be around 2% of the native enzyme. It is important to note that the assays were carried out in 50mM potassium phosphate buffer, *i.e.* in a buffer concentration that allowed the interaction between the domains but did not allow direct binding of cytochrome c with the FMN domain (Figures 4.16, 4.17). In order to test for the reconstitution of cytochrome P450 monooxygenase activity the rat cytochrome P450 CYP1A1 was incorporated into incubations containing the FMN/anchor and FAD/NADPH domains. Activity was monitored by

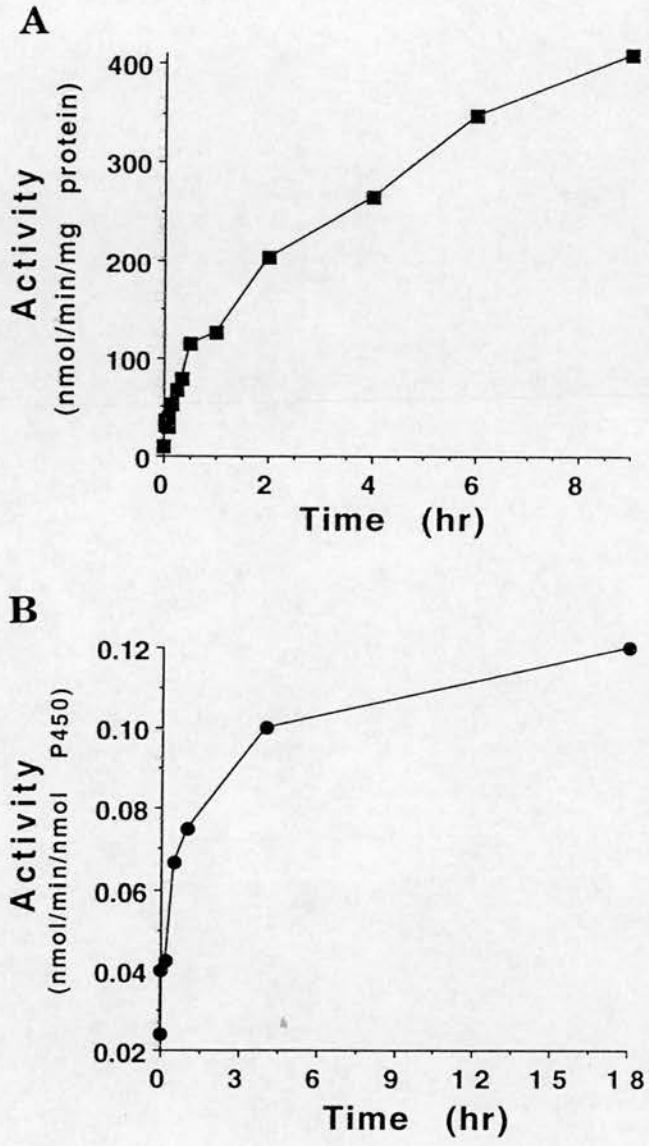



**Figure 4.18 Reconstitution of cytochrome c and cytochrome P450 reductase activity. (A)** Reconstitution of cytochrome c reductase activity using the FMN and FAD/NADPH domains. (0.1 nmol) of each domain were mixed in 10mM potassium phosphate buffer, pH7.7 in a volume of 20µl at 4°C, before assaying at various time points. **(B)** Reconstitution of cytochrome P450 reductase activity using the FMN/anchor and FAD/NADPH domains. (0.1 nmol) of each domain were mixed in 10mM potassium phosphate buffer, pH7.7 in a volume of 20µl at 4°C. After various times, the domains were incubated with dilaurylphosphatidylcholine (25µg) and CYP1A1 (37.5 pmol in 5µl) at 37°C for 5 minutes, before assaying for EROD activity by measuring the rate of resorufin formation.

measuring the rate of ethoxyresorufin O-deethylation (EROD) (Figure 4.18B). Using these two domains a functional monooxygenase system could be reconstituted that was time dependent and had a similar t1/2 to the reconstitution of cytochrome c reduction. (t1/2 estimations were kindly performed by Dr. David Tew, London). The FAD/NADPH domain alone had virtually no ability to reconstitute CYP1A1 monooxygenase activity (Table 1). These observations on the time dependence of reconstitution are intriguing. From the FMN and FAD/NADPH column binding experiment it was observed that the binding interaction was instantaneous and not time dependent. The time dependence observed in the reconstitution of activity may be due to establishing the correct orientation and/or stabilisation of the FAD and FMN for efficient electron transfer.

The reconstitution of P450 reductase activity using the FMN/anchor and FAD/ NADPH domains was also found to be ionic strength dependent (Figure 4.19). This result is in keeping with the observation that the FMN and FAD/NADPH complex was disrupted by increasing ionic strength.

The reconstitution of activities observed by the domains in this study, albeit extremely fascinating, may not optimal. As can be seen in Figure 4.2, the FMN and FMN/anchor domain share an exon 8 overlap with the FAD/NADPH domain. This may provide the two domains with some form of steric hindrance, resulting in the domains not interacting correctly with one another. Also, the FMN and FAD contents of the domains are below that found in native P450 reductase resulting in smaller specific activities than would be observed with domains containing the correct amount of flavin. Another important point for the FMN/anchor domain is the presence of a contaminating protein. Although this can be speculated to be a 10% contaminant it may have an effect on the reconstitution of cytochrome P450 reductase activity. One other factor that may be the cause of these interactions is the relative 'stickiness' of P450 reductase. This protein has 97 Asp and Glu residues and 74 Arg and Lys residues out of a total of 677 amino-acids. Therefore the specificity of the domain interactions that have been observed must be noted with caution.

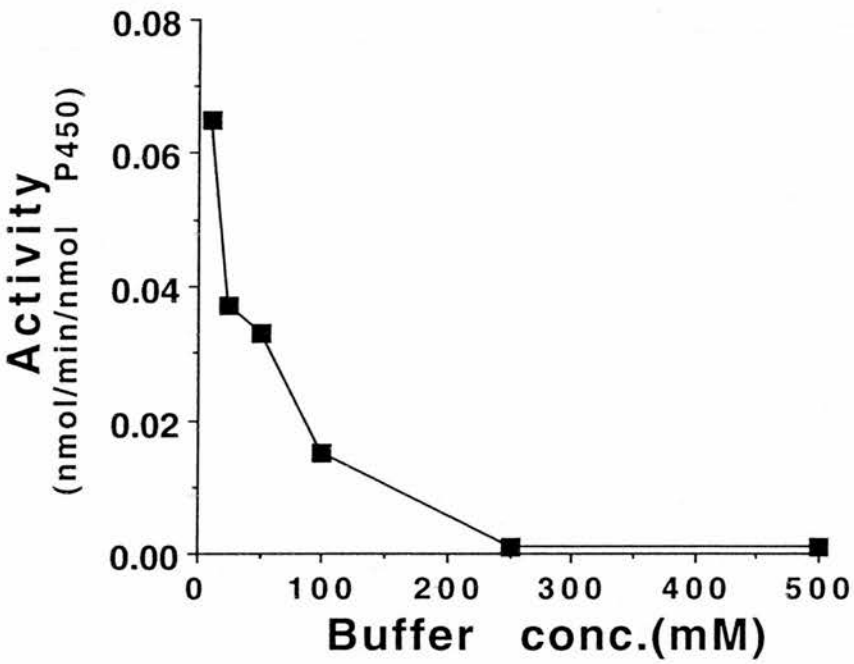



**Figure 4.19 Effect of ionic strength on the reconstitution of cytochrome P450 reductase activity.** The FMN/anchor and FAD/NADPH domains (0.1 nmol of each) were incubated for 2 hours in different concentrations of potassium phosphate buffer, pH 7.7 at 4°C. Dilaurylphosphatidylcholine (25µg) and CYP1A1 (37.5 pmol in 5µl) were then added. The samples were incubated for 5 minutes at 37°C before measuring EROD activity.

### 4.3.6. Effect of FMN, FMN/Anchor and FAD/NADPH domains on a reconstituted rat CYP1A1/ rat P450 reductase monooxygenase system.

The ability of the functional reductase domains to perturb a reconstituted monooxygenase system was looked at (Figure 4.20A, 4.20B). Perturbation of ethoxyresorufuin O-deethylation (EROD) activity produced by a reconstituted rat CYP1A1/ rat P450 reductase system was used. This assay is rapid, sensitive and highly specific for CYP1A1 (of which a large supply of purified enzyme was available in this laboratory).

Figure 4.20A shows the effect of the FMN/anchor domain and the FAD/NADPH domain on the reconstitution of EROD activity. By preincubating CYP1A1 with the FMN/anchor domain (for 5 minutes) almost complete inhibition of EROD activity was observed. Fifty percent inhibition was observed at a ratio of domain to native rat reductase of approximately 4:1. This inhibitory result could be explained by the domain preventing the association of the P450 to P450 reductase. This result is in keeping with a previous study where the purfied hydrophobic 6kDa. $NH_2$-terminal tryptic fragment from rabbit P450 reductase was found to be a potent inhibitor of reconstituted monooxygenase activity (Black *et al.*, 1979). However, the His-tag and thrombin cleavage site could not be removed for the FMN/anchor domain. It can not be ruled out that the His-tag may affect the association of the domain with the bilayer and that the inhibitory effect that is being observed may be occuring outside the bilayer. The FAD/NADPH domain had little effect on EROD activity, although a reproducible slight decrease in activity was seen at the lowest domain concentration tested (Figure 4.20A).

Most interestingly, the FMN domain was found to activate EROD activity by up to 3 fold (Fig. 4.20B). This appears to be specific for this domain as free FMN did not cause this effect . This activation effect could be explained if the FMN domain can accept electrons from the FAD of native P450 reductase and supply electrons directly to P450. Taken together with the results for the FMN/anchor domain (Fig. 4.20A) it can be suggested that the hydrophbic anchor is playing a role in the interaction of P450 with P450 reductase.

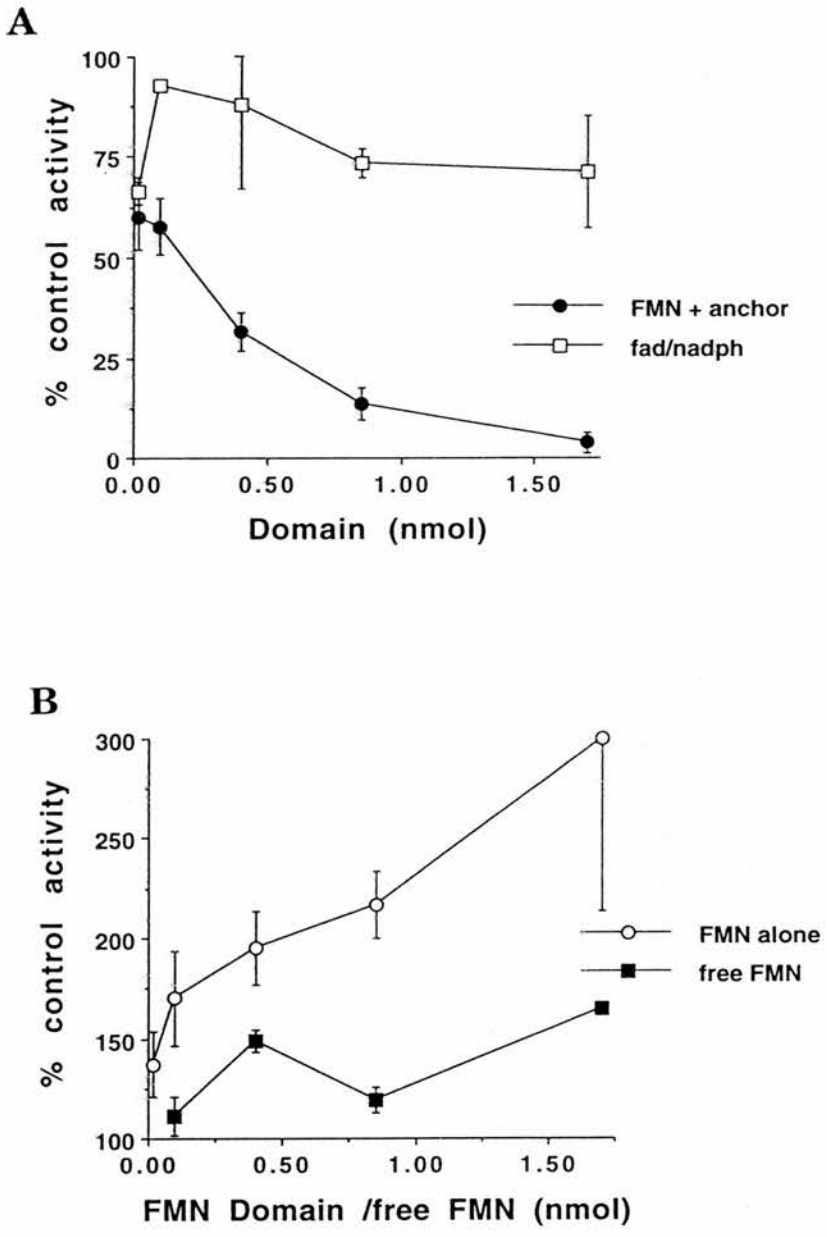



**Figure 4.20 Effects of the FMN/anchor, FMN, FAD/NADPH domains and free FMN on the reconstitution of monooxygenase activity.** CYP1A1 (7.5pmol) and 25µg of dilaurylphosphatidylcholine were incubated for 5 minutes with varying concentrations of **(A)** the FMN/anchor or FAD/NADPH domain (0.04-1.7 nmol) or **(B)** the FMN domain or free FMN (0.04-1.7 nmol) in a total incubation volume of 47.5µl at 37°C for 5 minutes. Native rat P450 reductase (0.05 nmol in 2.5 ml) was then added and the sample incubated for a further 5 minutes at 37°C before measuring EROD activity.

### 4.3 Summary and future work.

This chapter has described the dissection of human NADPH cytochrome P450 oxidoreductase into structurally and functionally independent domains. This work provides strong experimental evidence to substantiate the hypothesis that this protein has evolved from two distinct ancestral genes (Porter and Kasper, 1986). Indeed, many of the properties of the ancestral proteins appear to have been retained and there is no need for direct interaction between the two domains for FMN and FAD binding. However, for stabilisation of the incorporated flavin in the protein (i.e. to maintain a ratio of 1 molecule FMN or FAD to one molecule of protein) and for efficient electron transfer, interactions may be needed between the domains and/or flavins. It can be speculated that as two independent flavoproteins the ancestors of P450 reductase functioned as a dehydrogenase/ electron transferase in a primitive organism. By fusion of the genes encoding these ancient flavoproteins a more catalytically efficient electron transfer system was produced.

The FAD/NADPH domain could catalyse the reduction of potassium ferricyanide, dichlorophenolindophenol and menadione and showed transhydrogenase activity. These reaction rates were decreased by a variety of different levels compared to native P450 reductase but provides evidence that the FAD/NADPH domain is the site, at least in part responsible for the one-electron reduction of substrates.

The FAD/NADPH domain was able to be reduced by NADPH to a semiquione state which was not stable in air. This result was the same as that observed for FMN-depleted reductase (Vermillion and Coon, 1978b, Iyanagi *et al.*, 1981).

The FAD/NADPH domain was able to pass electrons from NADPH onto the FMN domain producing an air-stable semiquinone form similar to that seen for native reductase. When reduced by an excess of sodium dithionite the FMN domain produced an air-stable species that was identical to the one-electron reduced form of the FAD-depleted reductase (Kurzban *et al.*, 1990).

The FAD/NADPH and FMN domains could be reconstituted to form a complex that was active in the reduction of cytochrome c and in donating electrons for cytochrome P450 dependent monooxygenase activity. Although not optimal this result demonstrated that electron transfer between the domains and onto an electron acceptor could be achieved. The FMN domains and FAD/NADPH domain, however, had a 33 amino-acid overlap (exon 8).

This may have caused problems in the reconstitution of cytochrome c and P450 reductase activity by sterically hindering the correct interaction of the domains. Future work should focus at further minimilisation of the functional domains. Optimisation of domain size will also be of importance for future studies into the reconstitution of reductase activity. These should identify some of the structural and functional requirements needed for efficient transfer between the peptides. Removal of the contaminating factor from the FMN/anchor domain may also result in improved reconstitution. The results presented here have shown that it is not enough simply to express the exons that contain all the proposed amino-acids involved in binding FMN or FAD. Comparisons of the FMN binding region of P450 reductase with the flavodoxin from Desulfovibrio vulgaris show that highest homology lies within exons 4,5,6 (Porter *et al.*, 1990). This region, when expressed in *E. coli* did not bind flavin. However the larger FMN/anchor and FMN domains did bind flavin. Similarly, sequence alignments based on the crystal structure of spinach ferredoxin $NADP^+$ reductase reveal that the minimal FAD/NADPH binding region of the protein should be contained within exons 12-16 (Karplus *et al.*, 1991). However, expression of this small domain yielded an insoluble protein which, from its white appearance, did not bind FAD. In other cases, even when insoluble products were formed, the colour of the protein suggested flavin had been incorporated. It was a much larger FAD/NADPH domain, spanning exons 8-16 that was found to be functional.

Determination of the flavin content in the domains was found to be less than the predicted ratio of 1:1 mol protein: mol flavin. For the stabilisation of the flavin moieties in their respective domains, interactions maybe needed between the two domains. However, the reduced flavin content may be due to some incorrectly folded protein being produced by the bacteria or in the flavin being lost during the purification. Separation of the flavin and non-flavin containing domains may be possible since it is possible to purify out FAD depleted reductase from the holo-reductase (Kurzban *et al.*, 1990). To this end, some recent work in collaboration with Prof. Gordon Roberts, University of Leicester, has shown that the FMN containing domain can be separated from the non FMN containing protein by high resolution anion exchange chromatography. This should also be possible for the FAD/NADPH domain.

In conclusion; the data presented in this chapter support the hypothesis that P450 reductase has evolved as a fusion of two ancestral proteins (Porter and

Kasper, 1986, Porter *et al.*, 1990). Indeed, it can be speculated that as two independent proteins the ancestors of of P450 reductase functioned as a dehydrogenase/ electron transferase in a primative organism. By fusion of the genes a more catalytically efficient electron transfer system was produced. The ability to dissociate P450 reductase into domains will be of significant value for X-ray and NMR studies as well as in understanding about how the domains interact with each other. Future work may also look at the ability of the domains to interact with cytochromes P450 and cytochrome b5 to form a functional electron transfer unit. Such studies will not only provide new insights into the structure and function of P450 reductase but into other structurally related proteins such as nitric oxide synthase and sulfite reductase.

# Chapter 5: Purification and characterisation of a 59kDa. carboxylesterase from human liver microsomes and its role in the pathogenesis of halothane hepatitis.

## 5.1 Introduction.

The human microsomal carboxylesterases have been studied less in comparison to their rat counterparts. This is most probably due to the availability of tissue(s) for purification studies as is the case for other drug metabolising enzymes. Human liver microsomal carboxylesterases have been purified in the past using a variety of techniques and with varying degrees of success, (Jung *et al.*, 1974; Jung and Heymann, 1979; Tsujita and Okuda, 1984 and Ketterman *et al.*, 1989). Most of this previous work focused on the substrate specificity of the enzymes, molecular weight and isoelectric point. The early studies on the purified forms of the carboxylesterase either did not attempt to sequence the amino-terminus or found it to be blocked (Jung *et al.*, 1974; Tsujita and Okuda, 1984 and Ketterman *et al.*, 1989). Except for one human carboxylesterase, the 'low pI' form (Ketterman *et al.*, 1989), no amino-terminal sequence information has been elucidated from the purified enzymes. It was not until the cloning of a partial cDNA (Long *et al.*, 1989) and the recent full length cDNAs (Kroetz *et al.*, 1993) for human liver carboxylesterases that any sequence information for the human proteins has become available. Interestingly, the sequences obtained for the full length human carboxylesterases are 99.7% identical to that for a serine protease cloned from human liver macrophages (Munger *et al.*, 1991).

Interest in the microsomal carboxylesterases has risen in recent years as investigators have proposed a variety of roles for these enzymes other than their classical drug/xenobiotic metabolising role. Work by Mentlein *et al.*, (1988) has led to the suggestion that the hydrolytic activities of the carboxylesterases may facilitate the transfer of fatty acids across the endoplasmic reticulum or prevent the accumulation of potentially membrane lysing natural detergents. One of the rat liver microsomal carboxylesterases appears to be identical to egasyn, which functions to retain β-glucuronidase in the microsomal membrane (Medda, 1987). The major component of rat liver microsomal carboxylesterases, carboxylesterase E1/ hydrolase pI 6.0, has been shown to be involved in the non-oxidative metabolism of ethanol, fatty acid ethyl ester (FAEE) synthesis (Tsujita and Okuda, 1992). The role of

carboxylesterase in FAEE synthesis has been shown in a variety of tissues, especially adipose, lung and testis (Tsujita and Okuda, 1992). These workers purified an enzyme from rat adipose tissue with FAEE synthase activity and went on to show that the enzyme they had purified was the rat liver carboxylesterase pI 6.0 (Robbi *et al.*, 1990) also termed E1 (Harano *et al.* 1988). The identification of the carboxylesterase as FAEE synthase was based on enzyme activities, substrate specificities, inhibition studies and $NH_2$-terminal sequence.

As mentioned above, a serine esterase released by human alveolar macrophages was purified and cloned (Munger *et al.*, 1991) and shown to be identical to a partial human liver carboxylesterase cDNA (Long *et al.*, 1989). By aligning the alveolar serine protease with the recently cloned full length cDNAs, Kroetz *et al* (1993) showed that they are 99.7% identical and therefore most probably the product of the same gene. Munger *et al*, (1991), found their 60 kDa. serine protease to be the major esterase in the alveolar epithelial lining and that the protein was contained within and secreted by alveolar macrophages. In the alveolar lining layer there are numerous serine proteases capable of initiating or modulating the inflammatory process. For example, urokinase and the coagulation factor, factor VII are present and active in this layer (Chapman *et al.*, 1985 and Bowen *et al.*, 1985). Whether the carboxylesterase is involved in the modulation of the inflammatory process is unresolved. Another suggestion is that the enzyme acts as a detoxifier to air-borne environmental compounds (Munger *et al.*, 1991).

A proposed role for carboxylesterases has come from a study which showed that the elevation of cellular cysteine was mediated by hydrolysis of esters of cysteine (Butterworth *et al.* 1993). This is an important observation because of the potential of cysteine esters to increase cellular cysteine in order to protect against the toxicity of various chemicals (Lailey *et al.,* 1991, Butterworth *et al.*, 1993). An example being the ability of cysteine iso-propylester to protect against paracetamol hepatotoxicity.

The role of a microsomal carboxylesterase in the pathogenesis of the adverse drug reaction halothane hepatitis was put forward by Satoh *et al.*, (1989). Previous studies had found that patients with halothane hepatitis had antibodies in their sera that reacted with up to eight microsomal proteins of varying molecular weight (Kenna *et al.*, 1987a and Kenna *et al.*, 1988). By treating rats with halothane and then purifying halothane-modified proteins from the rat livers, Satoh *et al.* (1989) obtained a 59kDa protein. They then

went on to show that this protein was a previously characterised carboxylesterase. A variety of reasons why the carboxylesterase and other proteins are involved in the pathogenesis of this disease have been put forward. These include the ability of halothane to be modified to the reactive trifluoroacetylhalide metabolite, the relative abundance of the trifluoroacetylated proteins in the endoplasmic reticulum and the presentation of the modified proteins to the immune system. The studies using the rat carboxylesterase also showed that for patients' antibodies to react with the rat protein it had to be trifluoroacetylated. Reasons for the role of a carboxylesterase in halothane hepatitis will be discussed in more detail in section 5.3.1.

Therefore, previous work suggests that the microsomal carboxylesterase(s) are involved in a variety of biologically important reactions as well as being involved in the pathogenesis of an often fatal adverse drug reaction.

Tha aims of the work presented in this chapter were to characterise a 59kDa. carboxylesterase purified from human liver microsomes. The purification of this carboxylesterase will be presented along with the biochemical characterisation of the enzyme. The relationship of this carboxylesterase with the other forms of carboxylesterase that have been purified/ cloned in the past will be discussed. The role of the human microsomal carboxylesterase in the pathogenesis of the adverse drug reaction halothane hepatitis will also be considered. Using the sequences of the recently cloned human microsomal carboxylesterases (Kroetz *et al.*, 1993), computer generated predictions of some biochemical aspects of the protein will be outlined.

## 5.2 Results and Discussion.

### 5.2.1. Purification of a 59kDa. hepatic microsomal carboxylesterase.

A variety of methods have been employed in the purification of hepatic microsomal carboxylesterases. Solubilisation of microsomes has been overcome utilising the glycoside detergent saponin (Mentlein *et al.*, 1980 and Ketterman *et al.*, 1989), ionic detergents (Harano *et al.*, 1988) and non-ionic detergents, (Ozols, 1988). Early purification studies took into account the differences in isoelectric points of the proteins as a means of separation (Mentlein *et al.*, 1980, Mentlein *et al.*, 1987, Ketterman *et al.*, 1987). Indeed, some of the nomenclature for the carboxylesterases isolated to date have used

the pI values of the purified proteins (Mentlein *et al.*, 1980, Mentlein *et al.*, 1987). With regards to human carboxylesterase purification, Ketterman *et al.*, (1989) used a mixture of ion exchange chromatography, chromatofocussing and gel filtration to isolate their mid pI and low pI forms of carboxylesterase from saponin solubilised human liver microsomes. After the observation that carboxylesterases were glycosylated (Robbi and Beaufay, 1983), Harano *et al.*, (1988) employed concanavalin-A affinity chromatography into their protocol.

Since the purification described here was initially undertaken to obtain human NADPH cytochrome P450 oxidoreductase (for the production of rabbit polyclonal antisera) an initial step of ion exchange chromatography using DE52 was carried out (Yasukochi and Masters, 1976). This step was performed after the initial solubilisation of the microsomes with sodium cholate (Figure 5.1A). A pool containing a prominant 59 kDa. and 45 kDa. band, rich in cytochrome P450 as judged by spectral analysis, was obtained (Figure 5.1A, lane 3). This pool eluted in low (30 mM) potassium phosphate buffer, pH7.7 and then after dilution to 10 mM potassium phosphate, applied to a hydroxylapatite column and washed extensively. By stepwise elution the 45 kDa. band and the 59 kDa. band were separated at potassium phosphate concentrations of 20 mM and 60 mM respectively (Figure. 5.1B). The elution of cytochrome P450 pools followed at 100 mM potassium phosphate. (It should be noted that when purifying rabbit carboxylesterases Ozols, (1988) found that the protein co-purified with cytochromes P450 and epoxide hydrolase). At this stage in the purification described here the pure 45 kDa and 59 kDa. protein were unknown. It was not until $NH_2$-terminal sequence analysis was performed (Figure 5.3) that the 59 kDa. protein was identified as a carboxylesterase. The identification of the 45 kDa protein as epoxide hydrolase has been determined by Dr. J. G. Kenna, St. Mary's Hospital and Medical School, U.K. However, this is not the focus of any further work to be described in this thesis. Unfortunately protein elution profiles at 280nm could not be obtained because the non-ionic detergent emulgen 911, used throughout the purification, has an extremely high absorbance at 276 nm. A profile of carboxylesterase activity was not obtained since the protein was not identified as a carboxylesterase until late in the purification procedure. However, pools of the fractions obtained at various stages of the purification were kept and the activity towards the model carboxylesterase substrate p-nitrophenylacetate measured (Table 5.1). Previous work on the purification of

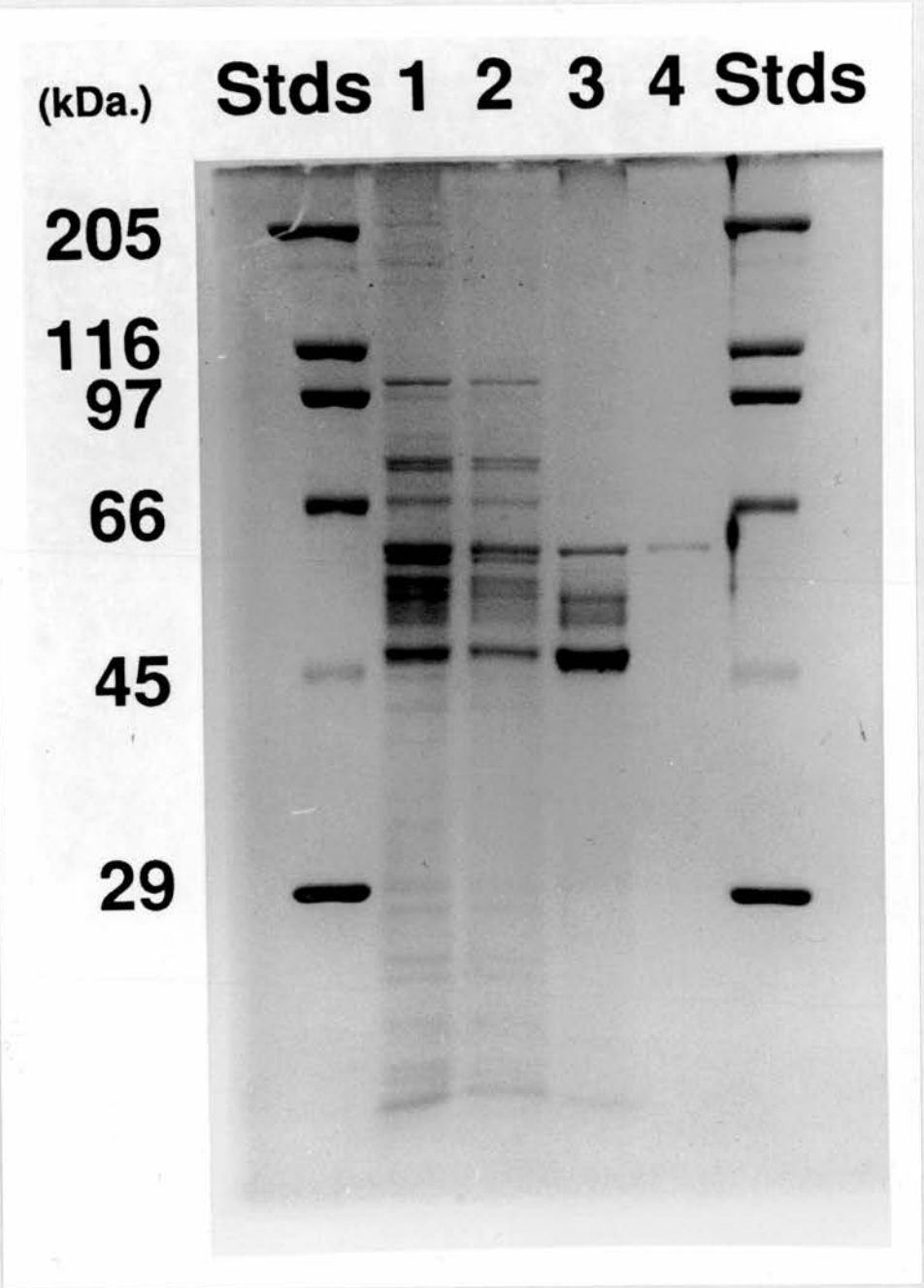



**Figure 5.1A.. Purification of a 59kDa. human hepatic microsomal carboxylesterase.** SDS-PAGE (9%) analysis of protein fractions obtained at different stages of the purification procedure. Lanes: 1, human hepatic microsomes (20μg); 2, solubilised human hepatic microsomes (20μg); 3, DEAE-cellulose carboxylesterase pool (10μg); 4, hydroxylapatite carboxylesterase pool (1μg)

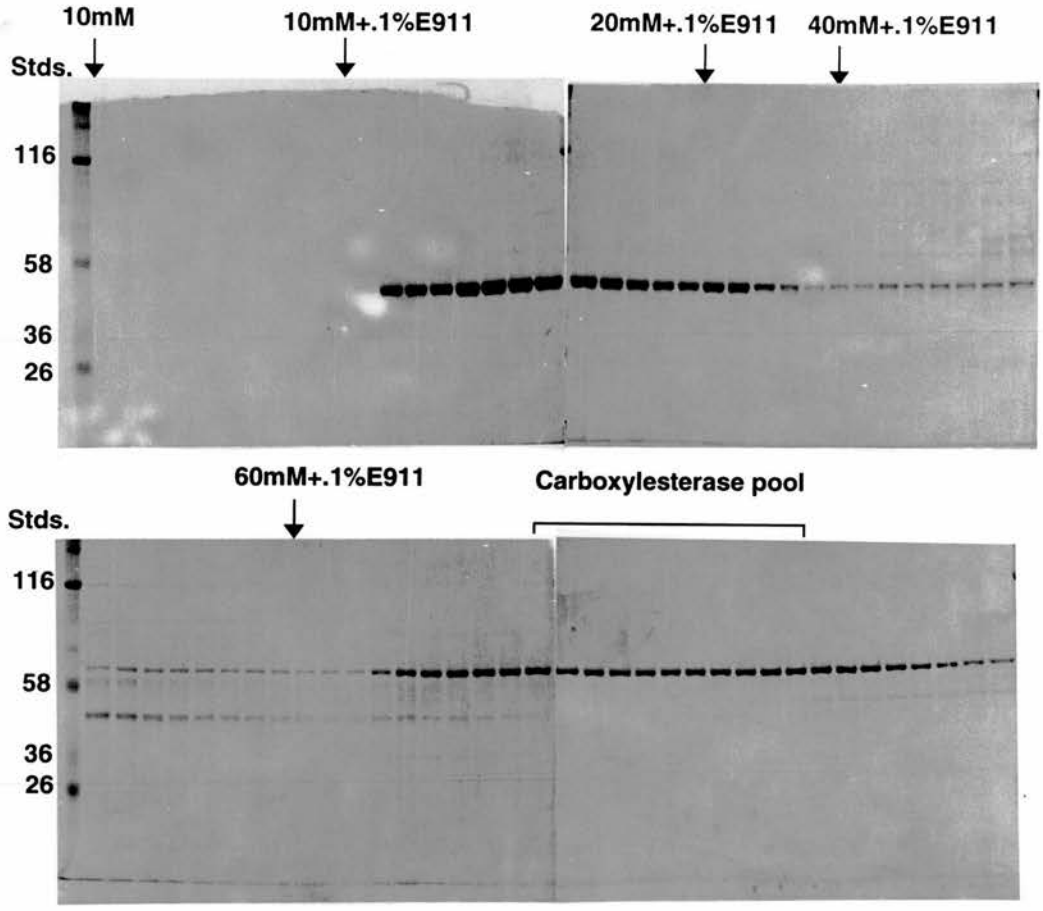



**Figure 5.1B. Purification of a 59kDa. human hepatic microsomal carboxylesterase.** SDS-PAGE (9%) analysis of fractions eluted from the hydroxylapatite stage.

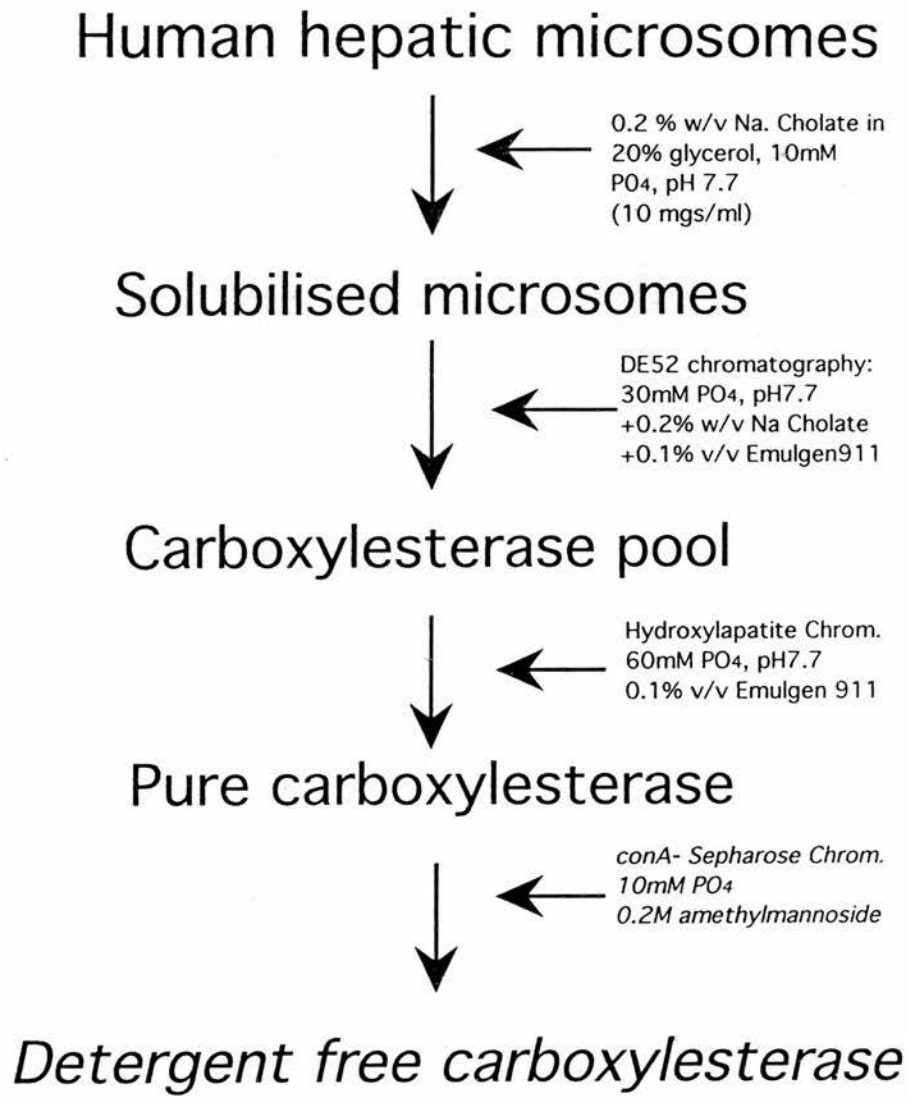



**Figure 5.2. Summary diagram of the human carboxylesterase purification.**

| Step | Protein (mg) | Total activity | S.A | Yield (%) |
|---|---|---|---|---|
| Microsomes | 9250 | 10452 | 1.13 | 100 |
| Sol. Micros. | 9250 | 8972 | 0.97 | 86 |
| DE52 | 2292 | 5517 | 2.25 | 53 |
| Hydroxylapatite | 32 | 678 | 21.2 | 6.5 |
| ConA-sepharose | n.d. | n.d. | 24.6 | n.d. |

Specific actvity (S.A.) was determined by measuring the rate of p-nitrophenyl acetate hydrolysis and is given as μmol product/min/mg protein.

n.d. not determined.

**Table 5.1 Summary table of the human microsomal carboxylesterase purification**

rat carboxylesterase (Harano *et al.*, 1988) had employed a concanavalin A sepharose step since the protein had been shown to be glycosylated (Robbi and Beaufay, 1983, Harano *et al.*, 1988 and Ozols, 1989). With this knowledge, concanavalin A chromatography was carried out on a fraction of the hydroxylapatite pool of carboxylesterase in order to remove the detergent Emulgen 911 and any other impurities (Table 5.1).

A summary of the purification is shown in Figure 5.2. The molecular weight of the carboxylesterase was determined as being 59 kDa. This is in keeping with the rat forms E1/ form pI 6.0 of the carboxylesterase (Robbi and Beaufay, 1983, Harano *et al.*, 1988; Hosokawa *et al.*, 1990) the baculovirus expressed human carboxylesterases (Kroetz *et al.*, 1993) and with the predicted size of the protein determined from its amino-acid sequence (Kroetz *et al.*, 1993). The native human 'mid pI' form of carboxylesterase has been shown to have a molecular weight of 173 kDa. (Ketterman *et al.*, 1989) This suggests the protein may exist as a trimer.

### 5.2.2. $NH_2$-terminal sequence analysis of the human microsomal carboxylesterase and alignment of other mammalian carboxylesterases.

After blotting the 59 kDa. protein onto a PVDF membrane, solid phase sequence analysis of the electroblotted protein (Matsuidara, 1987, Pappin *et al.*, 1990) was kindly carried out by Dr. Darryl Pappin, I.C.R.F., London. Difficulties were encountered in sequencing the $NH_2$-terminus of the protein, with a very low yield being obtained, suggesting blockage. However, some sequence was obtained as is shown in Figure 5.3. After a search of the OWL protein database, this was found to be highly similar to microsomal carboxylesterases purified from a variety of species (Figure 5.3). It was noted previously by Ketterman *et al.* (1989) that their human carboxylesterase 'mid-pI' form had a blocked $NH_2$-terminus, but the 'low pI' form was not. The sequence obtained here showed no similarity with the human 'low pI' form of carboxylesterase. However, no blockage of rat (Harano *et al.*, 1988; Hosokawa *et al.*,1989; Satoh *et al.*, 1989; ), rabbit (Ozols and Frankie, 1988; Ozols, 1989), hamster (Hosokawa *et al.*, 1990) or monkey (Hosokawa *et al.*, 1990) carboxylesterases has been reported. Alignments of the $NH_2$-termini obtained from a variety of mammalian microsomal carboxylesterases are

| | Residue Number | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| **Purified human protein** | Gly | Pro | Ser | Ser | Pro | Pro | Val | Val | Asp | Thr | | |
| **Human cDNA (Kroetz et al., 1993)** | His | Pro | Ser | Ser | Pro | Pro | Val | Val | Asp | Thr | Val | His |
| **Rat protein (Satoh et al., 1989)** | Tyr | Pro | Ser | Ser | Pro | Pro | Val | Val | Asn | Thr | Val | Lys |
| **Monkey (Hosokawa et al.,1989)** | Gly | Pro | Ser | Ser | Pro | Pro | Val | Val | Asp | Asp | Val | Lys |
| **Rabbit (Ozols, 1987)** | His | Pro | Ser | Ala | Pro | Pro | Val | Val | Asp | Thr | Val | Lys |
| **Rat cDNA (Takagi et al., 1988)** | His | Pro | Ser | Ser | Pro | Pro | Val | Val | Asp | Thr | Thr | Lys |

**Figure 5.3. NH2-terminal amino-acid sequence of the human carboxylesterase.** The top sequence is that of the data obtained in this study. References for the other enzymes are shown.

shown in Figure 5.3. The most striking observation from this is the high similarity between species.

### 5.2.3 The human liver microsomal carbxylesterase is glycosylated.

As is shown in Table 5.1, concanavalin A sepharose was used as an affinity step in the purification. Concanavalin A binds glycoproteins containing mannose and/or glucose hence it was assumed that these were the moieties bound to the human carboxylesterase described here. To further identify the carbohydrates bound to the carboxylesterase a glycan differentiation kit was employed (Figure 5.4). The results from this showed that the lectin Galanthus nivalis agglutinin (GNI) bound to the carboxylesterase. This lectin has been shown to bind to high mannose N-glycan chains on glycoproteins (Shibuya *et al.*, 1988).

To identify the quantity of carbohydrate groups bound to the carboxylesterase, the enzyme was treated with N-glycosidase F. This enzyme cleaves all types of asparagine bound N-glycans provided that the amino group as well as the carboxyl-group are present in a peptide linkage (Torentino *et al.*, 1985). On treatment with N-glycosidase F it can be observed that there is an increase in the mobility of the protein (Figure 5.5). A shift of around 2-3 kDa can be observed with some smearing being visible above the main band (Figure 5.5). The smearing most probably represents a mixture of partially deglycosylated forms of the carboxylesterase. Ozols (1989) observed a shift in the mobility of his rabbit carboxylesterases, forms 1 and 2, when he treated them with N-glycosidase F. The rat carboxylesterase E1 had a shift in electrophoretic mobility when digested with endoglycosidase H or α-mannosidase (Harano *et al.*, 1988). However, Ketterman *et al.*, (1989) showed that on treatment of their 'mid pI' and 'low pI' forms of human carboxylesterase with the glycosidases α-mannosidase and neuraminidase there was no effect on the banding pattern of the enzymes as judged by isoelectric focusing. Mannose residues have no overall charge, hence removal of them will not have any effect on the pI of the protein. Therefore, if mannose residues had been removed by the glycosidase treatment the banding pattern observed by iso-electric focussing would be no different from that seen with the native protein.

From the above data it can be concluded that the human microsomal carboxylesterase described here is a high mannose type glycoprotein containing up to an estimated 3 kDa. of sugar residues. Utilising a

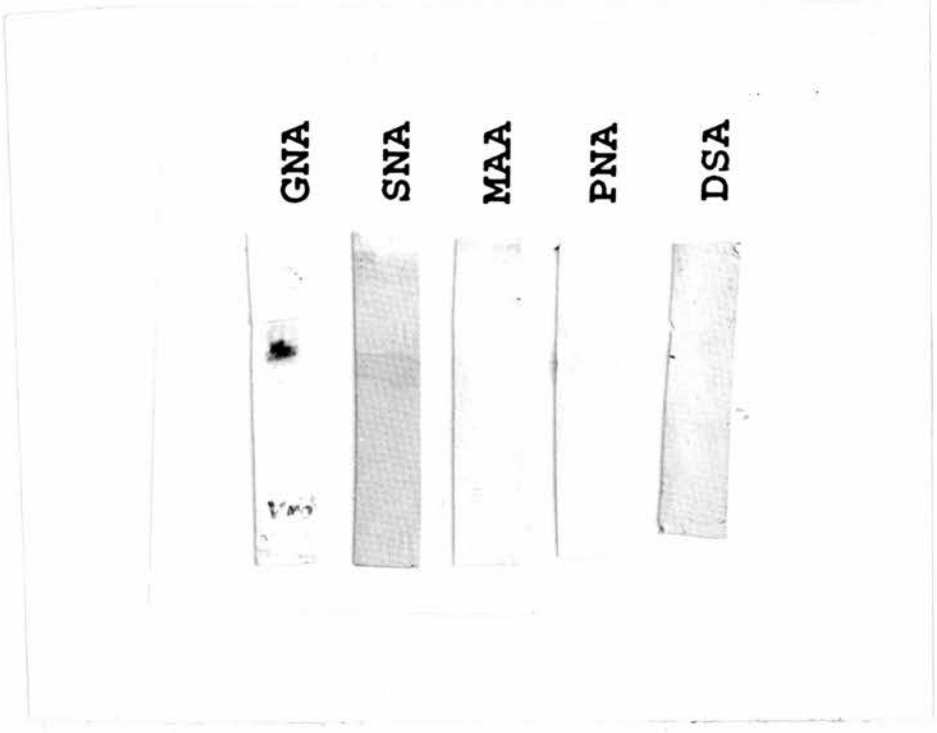



**Figure 5.4. Glycosylation status of the purified human carboxylesterase.** Purified carboxylesterase (2.5μg) was subjected to electrophoresis on a preparative 10-15% gradient SDS-polyacrylamide gel using the Pharmacia Phast System. The protein was then transferred onto a PVDF membrane which was subsequently cut into strips. Using a glycan dfferentiation kit, the carbohydrate residues on the carboxylesterase were identified as being mannose as judged by the binding of the lectin Galanthus nivalis agglutinin (GNI) to the carboxylesterase. Other lectins used were; SNA, sambucuc nigra agglutinin; MAA, Maackia amurenis agglutinin; PNA, peanut agglutinin; DSA, Datura stramonium agglutinin.

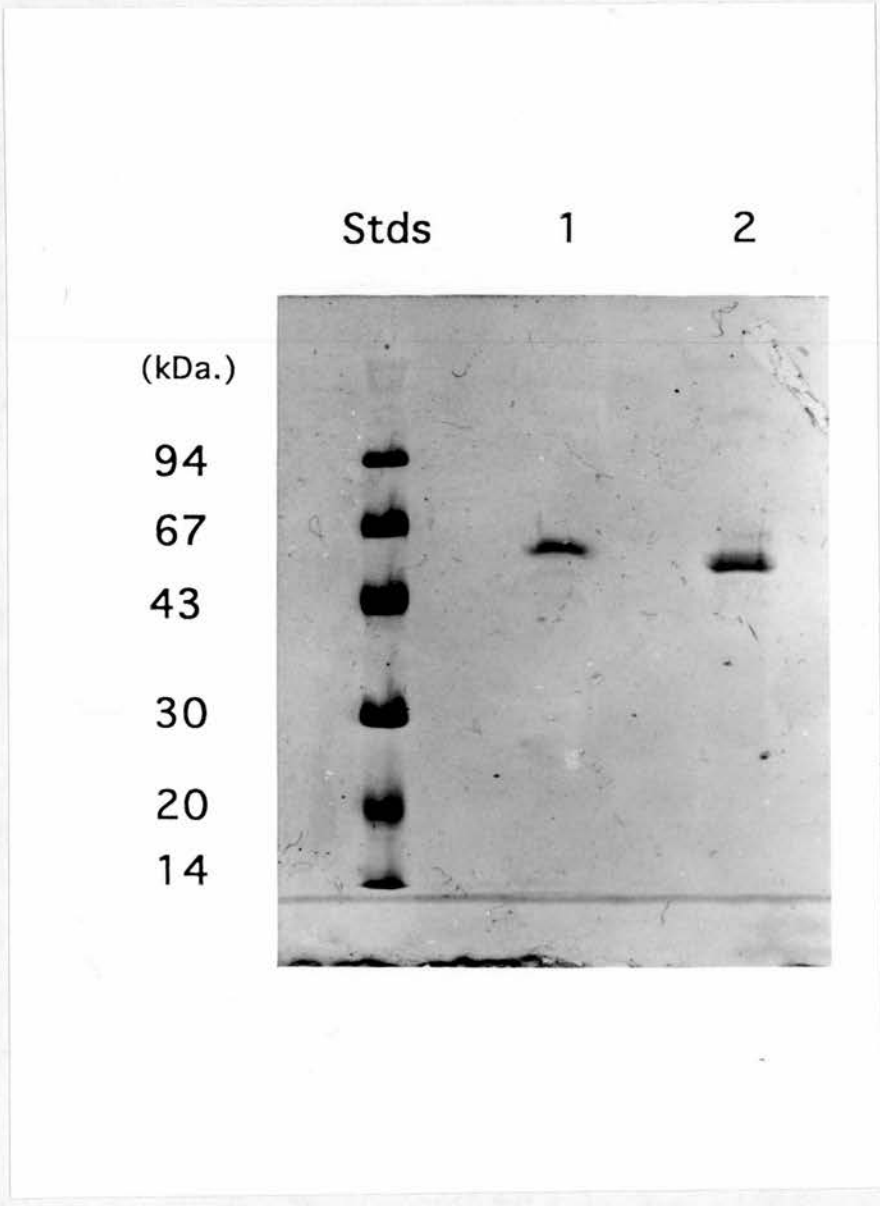


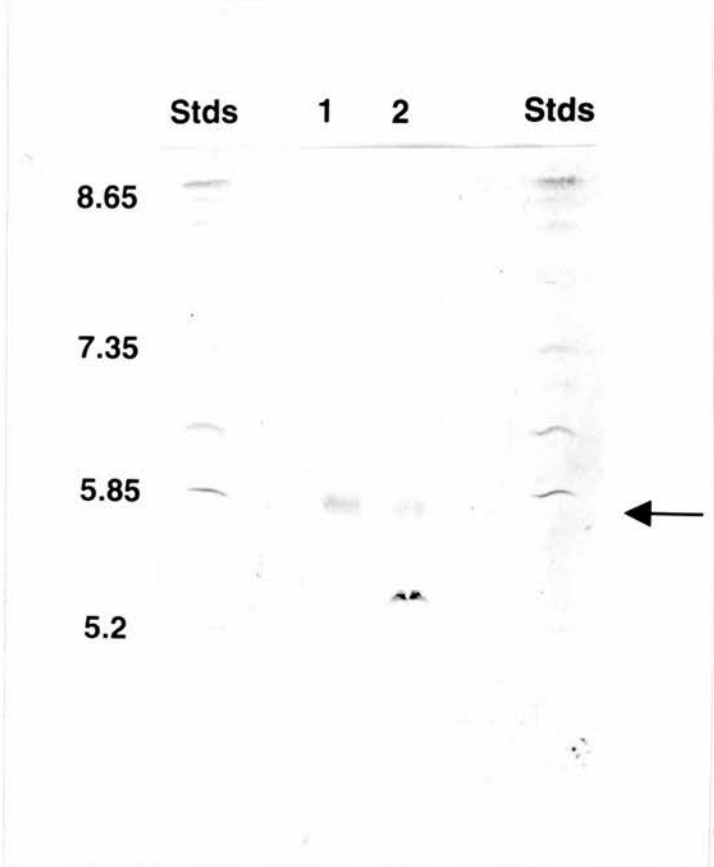

**Figure 5.5. Deglycosylation of the human carboxylesterase.** Pure carboxylesterase was digested with N-glycosidase F and the resulting products separated by SDS-PAGE (10-15% gradient gel, Pharmacia Phast System). Lanes: 1, Pure carboxylesterase (1μg); 2, N-glycosidase F treated carboxylesterase (1μg); Stds, molecular weight standards.

baculovirus mediated expression system, Kroetz *et al*, (1993) carried out experiments to look at the effects of glycosylation on the activity of the expressed carboxylesterase cDNAs. By inhibiting the glycosylation of their expressed protein they were able to show that glycosylation was important for the activity of the carboxylesterase towards a variety of substrates. Since N-linked glycosylation of proteins only takes place at the specific motif Asn-X-Thr/Ser (Hubbard and Ivatt, 1989) it is easy to predict potential sites of glycosylation on a protein if its sequence is known. The glycosylation site of the human carboxylesterases described by Kroetz *et al.*, (1993) is predicted to be at asparagine 62.

### 5.2.4 Isoelectric focussing of the human liver microsomal carboxylesterase.

Chromatofocussing and isoelectric focussing have played an important role in the purification and characterisation of carboxylesterases (Mentlein *et al*, 1980, Mentlein *et al.*, 1987, Ketterman *et al.*, 1989). For further characterisation of the carboxylesterase discussed here the determination of its pI was calculated both experimentally, using gel electrophoresis (Figure 5.6A), and by computation (Figure 5.6B) using the amino acid sequence predicted from the human liver microsomal cDNA of Kroetz *et al.*, (1993). Figure 5.6A reveals the carboxylesterase to have a pI value of approximately 5.8. The carboxylesterase bands are shown by the arrows. Samples were loaded from both the anode and cathode ends of the gel and shown to migrate to a similar point. The mark in lane 2 was an artifact from damaging the gel on loading the sample. The pI value obtained from the IEF gel is similar to that of the human 'mid pI' carboxylesterase determined by Ketterman *et al.*, (1989) as being 5.2-5.8. It is also similar to that of the purified rat carboxylestrase E1/ form pI 6.0 (Mentlein *et al.*, 1980, Harano *et al.*, 1988). Using the predicted amino-acid sequence from the cDNA of the human carboxylesterase (Kroetz *et al*, 1993) it was possible to predict the pI value of the protein using the computer program 'Isoelectric' from the GCG package. A plot of the result from the program is shown in Figure 5.6B. The total positive and negative charges and the net charge of the carboxylesterase are shown as a function of pH. The predicted pI value of the carboxylesterase was 6.46. This is higher than the experimental value of 5.8. This may be explained by the fact that the program assumes there are no electrostatic

A




B

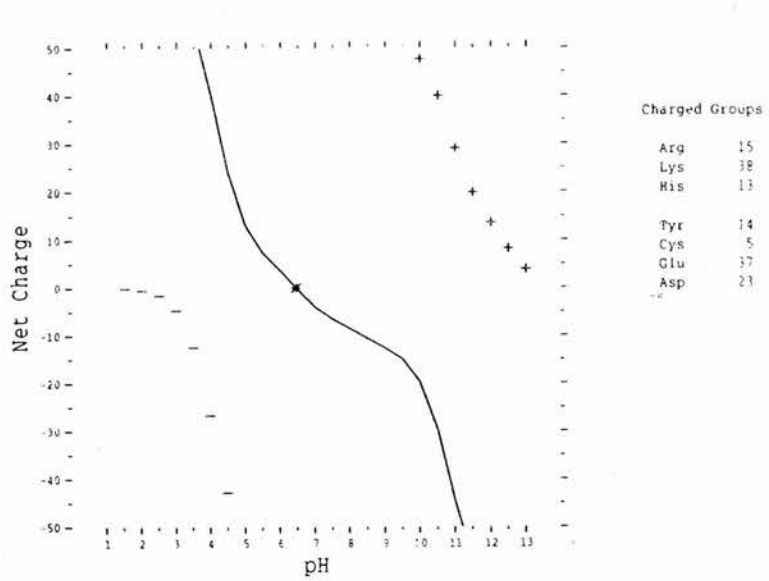



**Figure 5.6. Isoelectric point determination of the human carboxylesterase. (A)** Isoelectric focusing of the human carboxylesterase using a precast IEF gel from Pharmacia and performed on the Pharmacia Phast system. The arrow indicates the resolved carboxylesterase after two samples were loaded from either end of the gel. The lower dark band in lane 2 was obtained from damaging the gel on loading. **(B)** Computer prediction of the carboxylesterase isoelectric point using the program 'Isoelectric' from the GCG package.

interactions occuring within the protein that would perturb its overall ionisation. Of course the protein purified here may not be the form cloned by Kroetz *et al.*, (1993), however relative molecular weight, specific activities to the substrates shown in Table 5.2, glycosylation status and relative abundance leads me to believe that they are the same.

### 5.2.5 Activity of the human liver microsomal carboxylesterase.

The activity of the carboxylesterase towards a variety of substrates is shown in Table 5.2. The classic substrate p-nitrophenyl acetate was metabolised at a rate slower but in keeping with that observed by Ketterman *et al.*, (1989) for the 'mid pI' form of carboxylesterase, compare 21.2 μmol/minute/mg protein with 67.3 μmol/min/mg protein. The Km obtained was also similar to that reported previously, compare 0.18 mM with 0.13mM reported by Ketterman *et al.* (1989). The malathione data was also similar, compare specific activities of 0.42 with 2.79 μmols/ min/ mg protein. Since amidase activity of a purified human carboxylesterase has been reported previously (Junge and Heymann, 1979) the activity of the enzyme towards the arylamide substrates acetanilide and 4-nitroacetanalide was also looked at. However, the enzyme purified here was unable to hydrolyse these compounds. Amidase activity was not observed by Ketterman *et al*, (1989) for either the 'mid pI' or 'low pI' forms of human liver microsomal carboxylesterase. On studying the rat carboxylesterases it was found that amidase activity could only be observed for the low pI forms and not the mid pI forms (Mentlein *et al.*, 1987).

### 5.2.6. Inhibition of human liver microsomal carboxylesterase activity.

The ability of the serine protease inhibitor phenylmethylsulphonylfluoride (PMSF) (Gold, 1963) and the specific carboxylesterase inhibitor bis-4-nitrophenylphosphate (BNPP) to inhibit p-nitrophenylacetate hydrolysis by the human carboxylesterase was studied (Figure 5.7). p-Nitrophenylacetate hydrolysis was seen to be extremely sensitive to inhibtion by both PMSF and BNPP with $IC_{50}$ values of $2 \times 10^{-8}$ M and $2 \times 10^{-6}$ M respectively. Curve fitting of this data was kindly performed by Dr. David Tew, London. PMSF acts by reacting with the active site serine to give a sulphonyl enzyme derivative

| Substrate | Activity (μmols/min/mg protein) |
|---|---|
| p-nitrophenyl acetate | 21.4 |
| α-napthyl acetate | 11.0 |
| Malathione | 0.42 |
| Lambda cyhalothron (pyrethroid) | 0.052 |
| acetanilide | not detectable |
| 4-nitroacetoanilide | not detectable |

**Table 5.2. Specific activities of the human microsomal carboxylesterase.** The α-napthyl acetate, Malathione and lambda cyhalothron assays were kindly performed by Dr. Colin Walker, University of Reading.

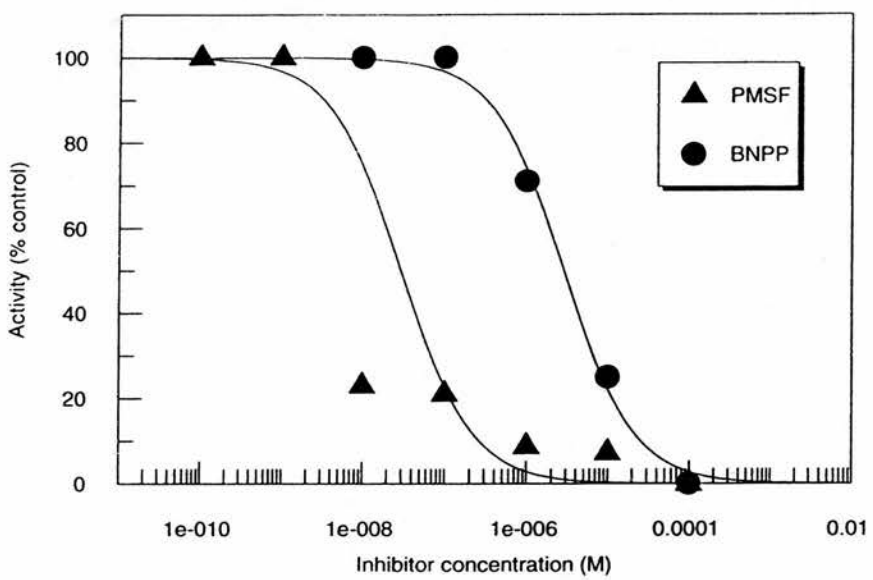



**Figure 5.7. Inhibition of carboxylesterase activity.** Inhibition of p-nitrophenylacetate hydrolysis by phenylmethylsulphonylfluoride (PMSF) and *bis*-4-nitrophenylphosphate (BNPP).

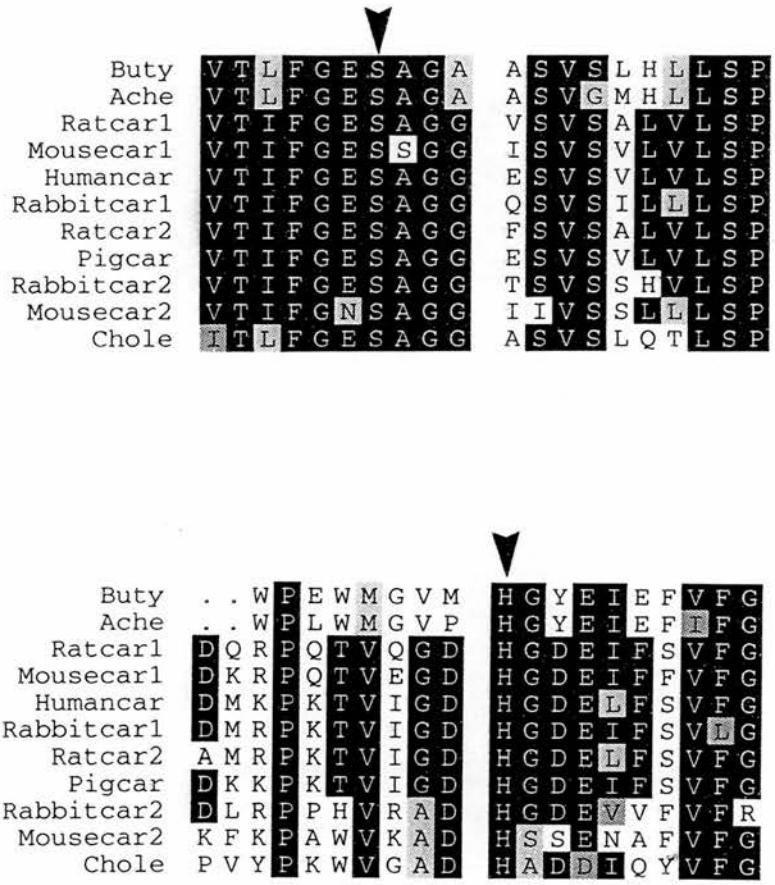



**Figure 5.8. Sequence alignment of a variety of esterases around the proposed active site residues.** This lineup was achieved using the programs 'Pileup' and'Prettybox' from the GCG package. The active site serine and histidine are marked with an arrow. Buty, human butyrylcholinesterase; Ache, human acetylcholinesterase; ratcar1, rat carboxylesterase E1 (major form); mousecar 1, mouse carboxylestersase form 1; humancar, human carboxylesterase; rabbitcar1, rabbit carboxylesterase form 1; ratcar2, rat carboxylesterase 2 (minor form); pigcar, domestic pig carboxylesterase; rabbitcar2, rabbit carboxylesterase form 2; mousecar2, mouse carboxylesterase form 2 and Chole, human cholesterol esterase.

which is resistant to spontaneous hydrolysis under neutral conditions (Gold, 1978). BNPP acts by irreversible phosphorylation of the active site serine (Brendt *et al.*, 1987). The active site serine and histidine for the rabbit form 1 carboxylesterase has been identified (Ozols, 1987). This has also been achieved for the related esterase enzyme, acetylcholinesterase (Schumacher *et al.*, 1987). Using the computer programs 'Pileup' and 'Prettybox', a multiple sequence alignment of a variety of esterases (obtained from the EMBL genebank) was produced (Figure 5.8) around these defined active site residues. Strong homology between liver microsomal carboxylesterases and other serine esterases can be seen to exist around the proposed active site serine and histidine residues.

### 5.2.7 Development of antisera to human microsomal carboxylesterase.

Polyclonal antiserum to the human carboxylesterase was raised in New Zealand white rabbits. (Figure 5.9) The antisera prepared allowed for Western blotting studies (Figures 5.10, 5.11, 5.12) and immunohistochemistry (Figure 5.16) to take place. The specificity and titre of the antibody for use in Western blotting studies is shown in Figure 5.9, using human liver microsomes and the purified human liver microsomal carboxylesterase. The antisera appeared to recognise a major band at 59 kDa. in human liver microsomes and a lower minor band when used at low dilutions (Figure 5.9).

### 5.2.8. Expression of the carboxylesterase in a human liver microsome panel.

Using the rabbit polyclonal antisera described in section 5.2.7. and sera from a patient with halothane hepatitis (see Figure 5.12, sera 2) the level of expression of the carboxylesterase was looked at using a previously characterised panel of human liver microsomes (Forrester *et al.*, 1992). It can be seen from Figure 5.10 that the level of expression across this panel is relatively constant and similar when contrasting the rabbit and human antisera. Densitometric scanning of the autoradiographs revealed the variation across the microsome panel to be 2.5 fold. This variation in the level of expression is virtually identical when comparing the rabbit anti-sera and the serum from the patient with halothane hepatitis. This small variation in

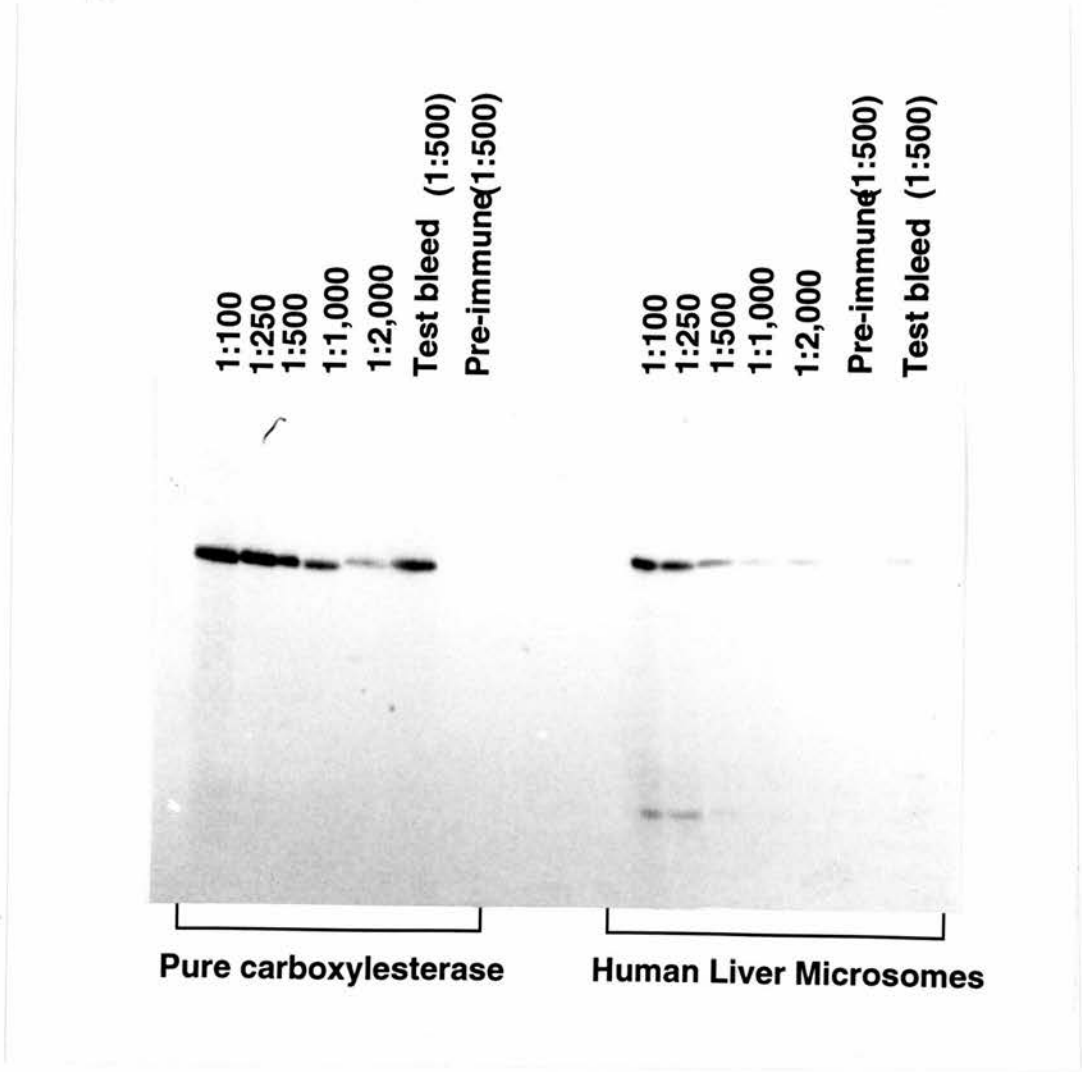



**Figure 5.9. Western blot analysis using rabbit polyclonal antisera raised to the purified human carbxylesterase.** The left hand panel shows a variety of dilutions used to probe purified carboxylesterase (200ng per lane) while human liver microsomes were used on the right hand panel (5µg per lane).

the level of carboxylesterase expression is in contrast to other drug metabolising enzymes that have been looked at using this panel (Forrester *et al.*, 1992). For example, some members of the P450 superfamily of drug metabolising enzymes were seen to vary by up to 60 fold (CYP4A1). By knowing the amount of protein loaded in the microsomal samples (7.5 μg) and that of the purified carboxylesterase (200ng) it was estimated from the densitometric scanning that the carboxylesterase makes up approximately 2% of total microsomal protein. The rat carboxylesterase E1/ form pI 6.0 has also been shown to be expressed at a high level in liver microsomes (Harano *et al.*, 1988). This study estimated that the rat carboxylesterase makes up to around 1.5% of the total rat endoplasmic reticulum protein. This high level of expression is most probably due to an endoplasmic reticulum retention signal KDEL found at the C-terminus of the protein (Pelham, 1991).

### 5.2.9 Expression of the carboxylesterase in other species.

Western blot analysis, using the rabbit polyclonal antisera, against a variety of microsomes from different species is shown in Figure 5.11A. This blot reveals that an immuno-reactive protein of similar molecular weight to the human carboxylesterase is present in rat, rabbit, dog, guinea pig and woodchuck liver microsomes, but not in mouse hepatic microsomes or in microsomes prepared form xenopus oocytes or yeast. A weak band does appear to be present in the lane containing the mouse microsomes, however this is very faint in comparison to the other mammalian species.

### 5.2.10 Expression of the human carboxylesterase in different human tissues.

Figure 5.11B shows the expression of the carboxylesterase in a variety of tissues by Western blotting using the polyclonal antisera described in section 5.2.7. Unfortunately only a small number of microsome samples could be obtained for this study. Figure 5.11B shows that this form of human microsomal carboxylesterase is only expressed at a detectable level in the liver microsome sample. It would have been interesting to look for expression of the carboxylesterase in adipose tissue since it has been shown that the major form of the rat microsomal carboxylesterase is expressed at a

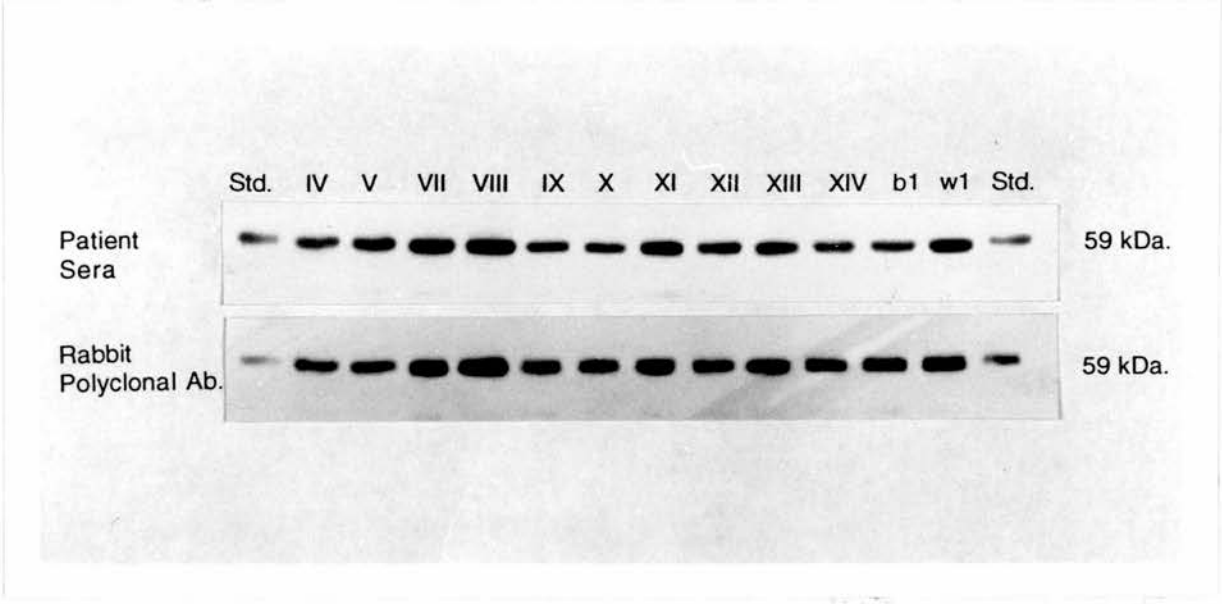



**Figure 5.10. Expression of the carboxylesterase in a human liver microsome panel.** Western blot analysis was performed using the rabbit antisera and serum from a patient (HH2, Figure 5.13) with halothane hepatitis. Lanes IV-w1, 7.5μg of microsomal protein. std, 200ng purified carboxylesterase.

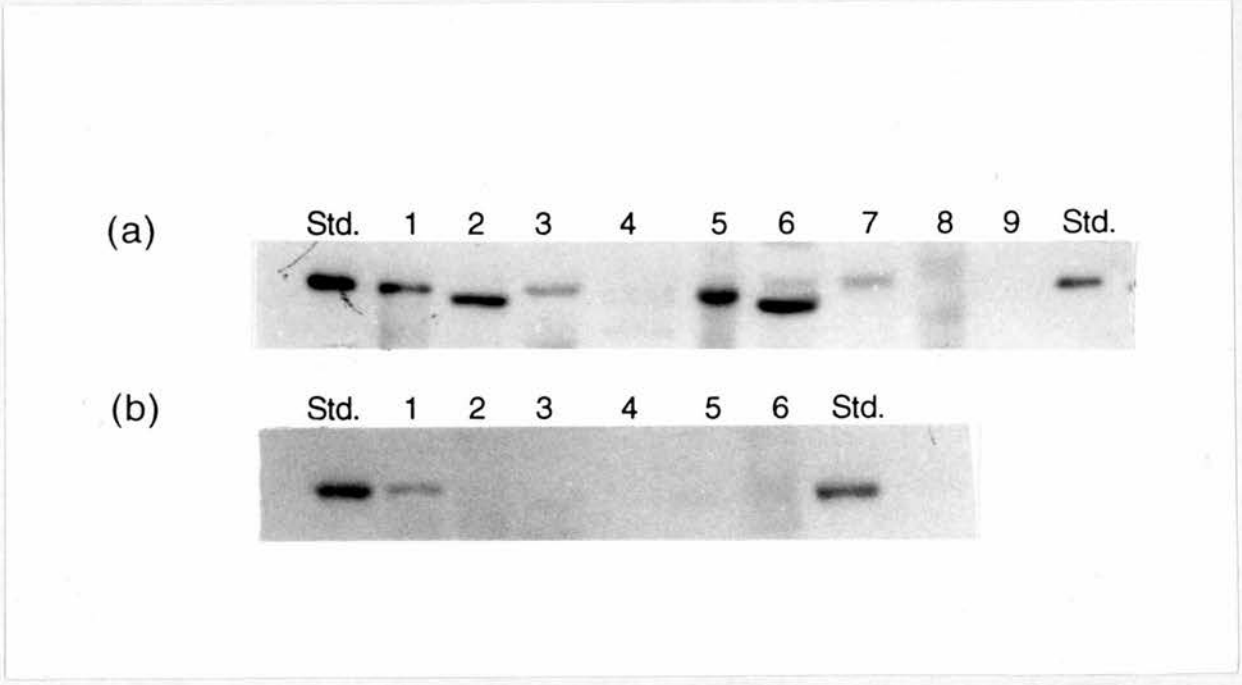



**Figure 5.11. Expression of a 59 kDa. carboxylesterase in (A) other species and (B) in human tissues.** **(A)** Lanes 1-7 (liver microsomes): 1, human (1 μg); 2, rat (10 μg); 3, rabbit (10 μg); 4, mouse (10 μg); 5, dog (10 μg); 6, guinea pig (10 μg); 7, woodchuck (10 μg) and microsomes from; 8, xenopus (75 μg) and 9, yeast (75 μg). Std, 200ng pure human carboxylesterase. **(B)** Lanes 1-5 human microsomes from 1, liver (1 μg); 2, kidney (50 μg); 3, colon (50 μg); 4, bladder (50 μg); 5, ovaries (50 μg); 6, testis (50 μg); std, 200ng pure human carboxylesterase.

high level in this tissue and is involved in fatty acid ethyl ester synthesis (Tsujita and Okuda, 1992)

## .5.3 The role of human liver microsomal carboxylesterase in the pathogenesis of halothane hepatitis.

### 5.3.1. Introduction to halothane hepatitis.

Halothane has been widely used as an inhalation anaesthetic agent since its introduction in 1956 (Johnstone, 1956). Halothane can cause two types of hepatotoxicity. The milder form is characterised by minor elevations in serum transaminases, develops in around 20% of patients and has been attributed to cytotoxic effects of halothane metabolites (Ray and Drummond, 1991; Kenna *et al.*, 1993). The more severe form of hepatotoxicity, termed 'halothane hepatitis', is much rarer, affecting 1 in 10,000, and results in severe hepatic damage (Ray and Drummond, 1991). Halothane hepatitis is characterised by extremely large elevations in serum transaminase levels, jaundice and massive hepatic necrosis with a significant fraction of patients developing liver failure which is most often fatal (Zimmerman, 1978; Ray and Drummond, 1991). Halothane hepatitis is an idiosyncratic and dose independent adverse drug reaction. However, patients who develope this disease are frequently female, obese and of late middle age (Zimmerman, 1978; Ray and Drummond, 1991). From the clinical and laboratory features of the hepatitis it has been implied that it is of an immunological basis. For example, all patients have been exposed to halothane on multiple occasions with a substantial proportion having developed an adverse reaction, such as fever or jaundice, after a previous exposure (Zimmerman, 1978; Walton *et al.*, 1978; Neuberger and Kenna, 1987; Ray and Drummond, 1991). Another important observation has been that the inter-operative interval can be prolonged, ranging from 7 days to 10 years (Neuberger and Kenna, 1987). Compared to direct hepatotoxins, such as paracetamol, the interval from the anaesthesia to the onset of liver dysfunction is delayed by up to 25 days in some cases (median 7 days), (Kenna *et al.*, 1987b). These observations were the basis for the hypothesis that halothane hepatitis is mediated through an immune response. Other observations suggesting that an immune reaction is the cause of the disease include pyrexia, eosinophilia and elevated

circulating immune complexes (Zimmerman, 1978; Walton *et al.*, 1976; Neuberger and Kenna, 1987). Importantly, it has been discovered that there are circulating antibodies in the sera of halothane hepatitis patients that are directed against halothane modified liver antigens (trifluoroacetylated (TFA) proteins), (Kenna *et al.*, 1987a; Kenna *et al*, 1988; Kenna *et al*, 1993). This latter point has led to the hypothesis that metabolites of halothane react with proteins within the cell to produce proteins that are recognised as non-self by the immune system in susceptible individuals (Pohl *et al*, 1989; Kenna *et al.*, 1993). Under oxidative conditions halothane is metabolised to a trifluoracetyl radical which can interact with lysine residues of proteins, resulting in trifluoroacetylated (TFA)-proteins. (Harris *et al.*, 1991). These TFA-proteins have been proposed to go on to elicit an immune response which can cause immune mediated hepatic necrosis in susceptible individuals (Pohl *et al.*, 1989; Kenna *et al.*, 1993). In recent years, much research has gone into identifying what these halothane modified proteins are. Western blot analysis reveals that up to eight different proteins in the microsomal fraction of livers from rats treated with halothane are recognised by antibodies present in the sera from halothane hepatitis patients (Kenna *et al.*, 1987; Kenna *et al.*, 1988). These proteins have varying molecular weights of 100, 80, 63, 59, 58, 57 and 54 kDa. Recent work in identifying these proteins has focussed around purifying the TFA-proteins from the liver microsomes of halothane treated rats. The first protein to be identified was the 59 kDa TFA-protein (Satoh *et al.*; 1989). This protein was found to be a previously characterised hepatic microsomal carboxyesterase, form E1/ pI 6.0 (Harano *et al.*, 1988). Since this initial identification, the other TFA-proteins that have been isolated and identified are the 100kDa. protein; GRP94/ ERp99/ endoplasmin (Thomassen *et al.*, 1990), the 80kDa. protein; ERp72 (Pumford *et al.*, submitted), the 63kDa. protein; calreticulin (Butler, 1992) and the 57kDa. protein; protein disulphide isomerase (Kenna *et al.*, 1990)

From the $NH_2$-terminal sequence of the human carboxylesterase (Figure 5.3), molecular weight (Figure 5.1A,B), the isoelectric point (Figure 5.6) and the specific activity towards carboxylesterase substrates (Table 5.2) it is clear that the human carboxylesterase purified here is or is highly similar to the enzyme purified form halothane treated rat livers (Satoh *et al.*, 1989). Satoh *et al.*, (1989) proposed that a human form of the carboxylesterase would be a target for the antibodies found in halothane hepatits patients' sera. In order

to test this hypothesis out a variety of experiments were carried out which are described in the following sections.

### 5.3.2. Detection of antibodies to human microsomal carboxylesterase in halothane hepatitis patients' sera by Western blotting.

The aim of the experiment shown in Figure 5.12 was to see if any patients had antibodies in their sera directed against purified human carboxylesterase that could be detectable by Western blotting. It is important to note that the carboxylesterase purified here was from a pool of liver microsomes obtained from organ transplant donors who had had no previous exposure to halothane. Therefore, it could be assumed that the carboxylesterase was not trifluoroacetylated.

Previous studies using sera from patients with halothane hepatitis have used the method of Western blotting to identify proteins that are the targets for antibodies found in the sera (Kenna *et al.*, 1987a, Kenna *et al.*, 1988, Satoh *et al.*,1989; Kenna *et al.*, 1993). These studies have relied on using hepatic microsomes from halothane treated rats or rabbits in order to identify TFA-proteins recognised by the antibodies in the patients' sera. Sera from 20 patients with halothane hepatitis, kindly provided by Dr. J. Gerry Kenna, St. Mary's Medical School, London, were studied. These patients were defined clinically as having otherwise unexplained hepatitis within 25 days of halothane anaesthesia. Four control samples from patients who had recieved multiple exposures of halothane but with no adverse reaction to the drug were also used. Figure 5.12 shows that only one patient (HH 2) had detectable amounts of antibody in their sera directed against the purified carboxylesterase when using a serum dilution of 1:100 with the secondary antibody at a dilution of 1:1000. All other patients' sera appeared not to contain antibodies directed against the carboxylesterase that could be identified by this form of immunodetection. However, this result is the first strong evidence of autoantibodies against the carboxylesterase in a patient with halothane hepatitis, *i.e.* the antibodies present in patient HH2's sera are true autoantibodies and not antibodies against the trifluoracetylated form of the carboxylesterase. When using native rat carboxylesterase in comparison to rat TFA-carboxylesterase Satoh *et al.* (1989) found that for patients' sera to react with the carboxylesterase it had to be modified by halothane. In another

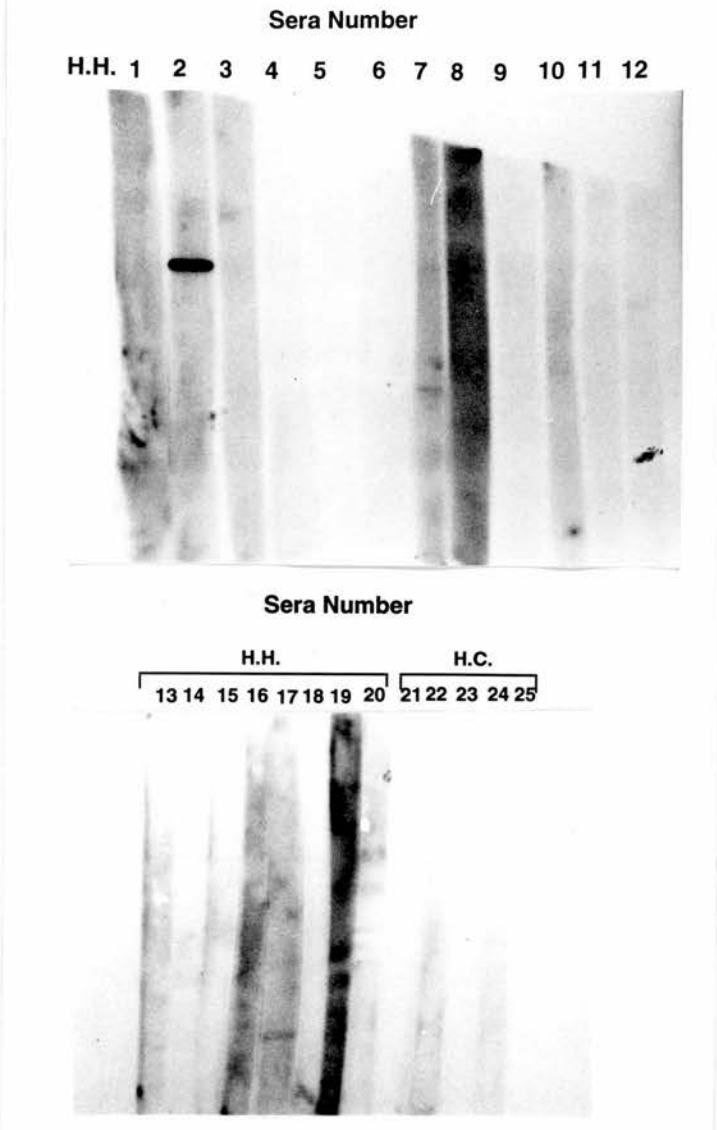



**Figure 5.12. Detection of antibodies to human microsomal carboxylesterase in halothane hepatitis patients' sera by Western blotting.** After tranferring purified carboxylesterase from a 9% preparative poly-acrylamide gel to nitrocellulose, the membrane was cut into strips (~ 200ng protein/ strip) and Western blot analysis was carried out. Lanes 1-20, halothane hepatitis patients' sera. Lanes 21-25, control sera (patients exposed to halothane but with no adverse reaction).

study (Kenna *et al*, 1988) the general rule found was that patients' antibodies did not recognise proteins in liver microsomes from untreated rats. Therefore previous studies have concluded that the antibodies present in patients' sera were directed against TFA-proteins, not wild-type proteins. This appears not to be the case for patient HH2 (Figure 5.12). This may be due to the fact that this is the first time a human protein has been used for this sort of study. Previous work has only ever utilised rat or rabbit protein samples. At the amino acid level the rat carboxylesterase form E1/ pI 6.0 is only 77% identical to the human carboxylesterase as predicted from the computer algorithm 'Bestfit'. This lack of sequence homology suggests that epitopes found on the native human carboxylesterase, by antibodies in the patient's serum, may not be present on the native rat or rabbit protein.

### 5.3.3 Detection of antibodies to human microsomal carboxylesterase in halothane hepatitis patients' sera by dot-blotting and ELISA.

Since Western blotting is carried out under denaturing conditions then conformational epitopes on proteins may be destroyed by the harsh conditions used in this method. In order to study whether the patients' sera contain antibodies to epitopes not detectable by Western blotting the non-denaturing methods of dot-blotting and ELISA were carried out. Figure 5.13 shows the result obtained for the detection of antibodies in patients' sera using a dot-blot. In this experiment 20 patients diagnosed as having halothane hepatitis were used alongside 6 control sera from patients who had recieved halothane on multiple occasions without an adverse reaction and 10 control sera from healthy blood donors. This blot clearly demonstrates that 7/20 halothane hepatitis patients have antibodies in their serum directed against the native carboxylesterase (patients HH2, HH6, HH7, HH8, HH16, HH17, HH20). Low levels of antibodies were detected in patients HH9, HH10 and in the controls HC 1, HC2 and BD3, BD4, BD7, BD8. The significance of these control results are unclear and may be due to non-specific binding of antibodies and/or protein A to the filter. To obtain a more quantitative analysis of antibodies present in patients sera an ELISA was set up (Figure 5.14). Due to the ease of this assay a larger range of control samples could be analysed. This control pool comprised of 9 sera from patients who had recieved multiple exposures of halothane but with no adverse effects, 18 healthy blood donors, 11 patients

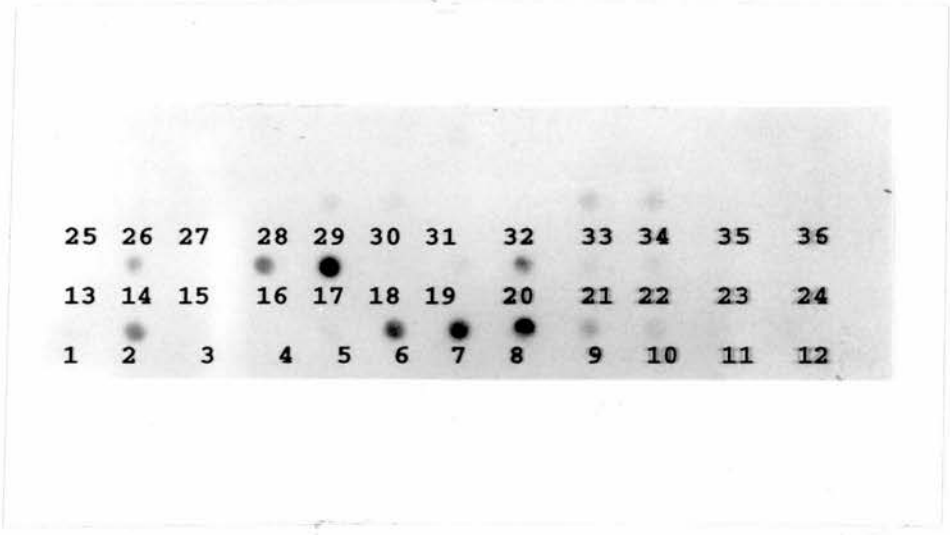



**Figure 5.13. Detection of antibodies to human microsomal carboxylesterase in halothane hepatitis patients' sera by dot-blotting.** 100ng of protein per dot was probed with patients' sera. Dots 1-20 (HH1-20), halothane hepatitis patients' sera; Dots 21-26, halothane control sera (HC1-6)., 27-36, healthy blood donor sera (BD1-8).

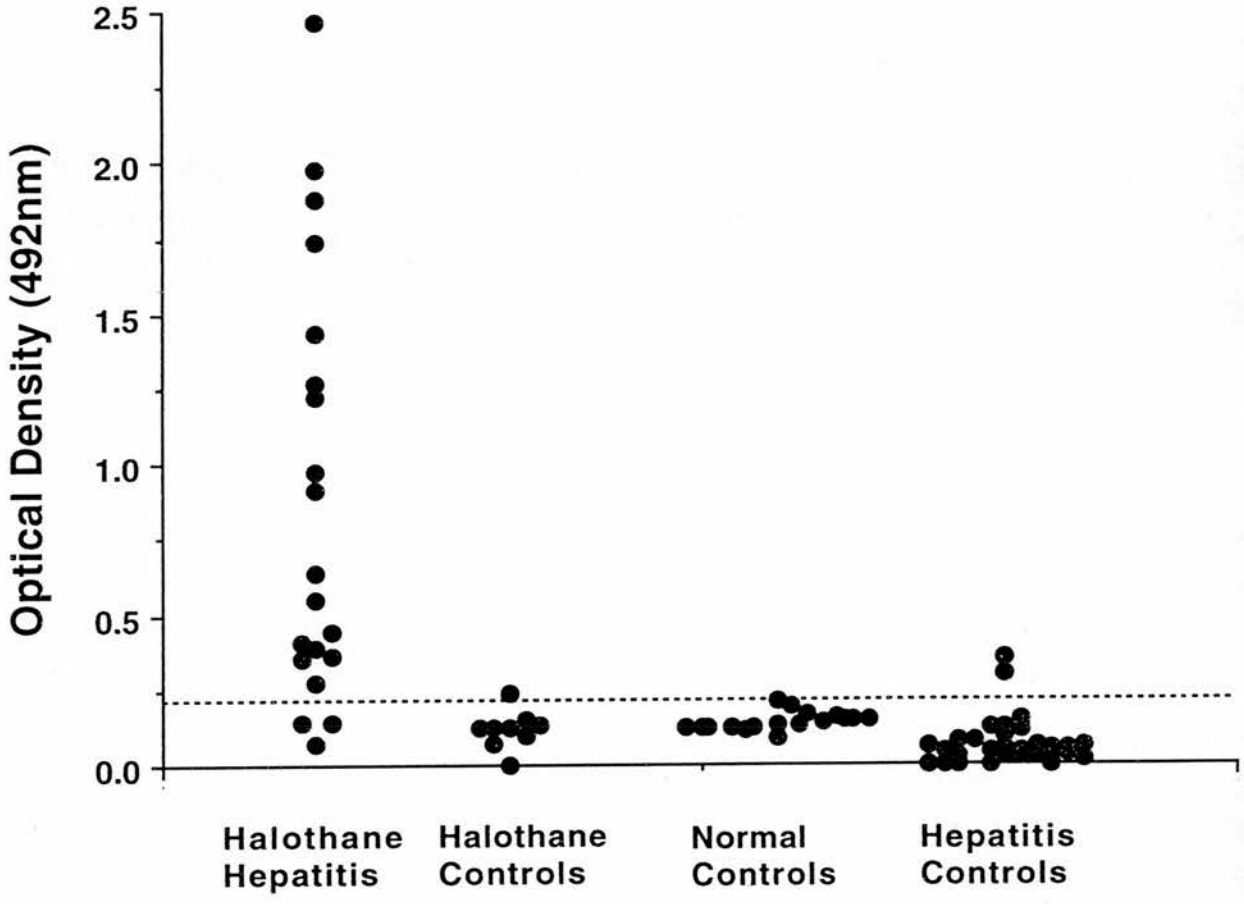



**Figure 5.14. Detection of antibodies to the purified human carboxylesterase in patients' sera by ELISA.** Dotted line shows upper limit of the normal control range. O.D., optical density.

with fulminant hepatic failure (6 paracetamol, 2 hepatitis B, 1 hepatitis A, 2 non-A and nonB hepatitis), 6 with autoimmune chronic active hepatitis, 4 with alcohol liver disease, 8 with primary biliary cirrhosis and 4 with jaundice due to flucloxacillin. Figure 5.14 shows the result of the ELISA assay using sera at a dilution of 1:100 and screened using HRP-conjugated goat anti human IgG antisera at a dilution of 1:1000. Sera were regarded as antibody positive when the optical density (O.D.) values at 492nm were greater than 2 standard deviations above the mean value for the healthy blood donors (O.D. > 0.211). Antibodies to human liver carboxylesterase were detected in the sera of 17/20 patients with halothane hepatitis but not in the 9 halothane exposed controls or 31 of the 33 'other hepatitis' control patients. Interestingly, low levels of antibodies to the carboxylesterase were detected in 2 of the 8 patients with primary biliary cirrhosis (PBC). This may be explained by the fact that in PBC an autoimmune response is mediated against a very wide range of proteins. The carboxylesterase may have been one of these in the patients looked at here. Alternatively, a similar epitope may have been recognised and hence the nature of this result may be one of non-specificity. An interesting observation is that sera HH2, which recognised the carboxylesterase by Western blotting (Figure 5.12), did not have the highest titre (sera HH8 had the highest titre in this study). This can be explained by the fact that ELISA allows the detection of conformational epitopes which may be lost through the harsh conditions of Western blotting. The ELISA data (Figure 5.14) reveals the presence of autoantibodies against human microsomal carboxylesterase in 85% of patients tested with halothane hepatitis. This provides the first clear evidence that autoantibodies against a human microsomal protein are present in the sera from patients with halothane hepatitis. Previously, it has always been assumed that the antibodies found in the patients' sera are against neo-antigens, i.e. trifluoroacetylated regions of the protein or novel haptens produced on the protein via the chemical modification but distinct from sites of trifluoroacetylation. (Kenna *et al.*, 1988; Satoh *et al.*, 1989)

### 5.3.4 Immunohistochemical localisation of microsomal carboxylesterase in human and rat liver sections.

An early observation on the pattern of hepatic damage caused by halothane hepatitis was that it was predominantly centrilobular in nature (Peters *et al.*,

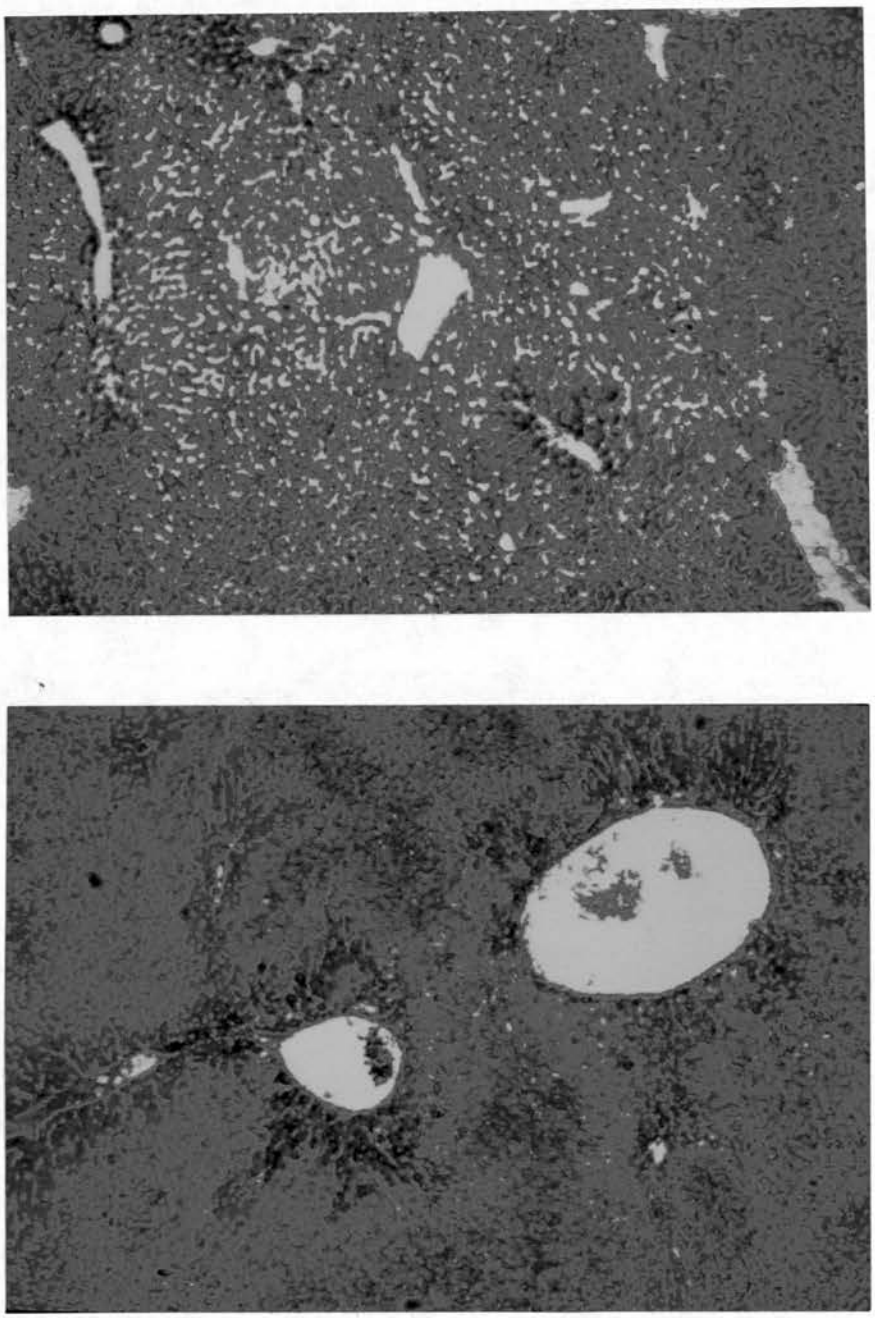


**Figure 5.16. Immunohistochemical localisation of microsomal carboxylesterase in (A) a human liver section and (B) a rat liver section.** Blue signifies highest antibody binding with red representing weaker regions of binding.

1969; Benjamin *et al.*, 1985). Using the rabbit polyclonal antisera raised against the human carboxylesterase (Figure 5.9) the localisation of the carboxylesterase was looked at in both human and rat liver sections. This work was carried out in collaboration with Dr. David J. Harrison, Pathology Dept., University of Edinburgh. False colour enhanced immunohistochemistry was performed with blue representing the region of highest antibody binding with green through to red indicating weaker regions of binding. Figure 5.15A reveals that the human carboxylesterase and its rat counterpart (Figure 5.15B) are localised predominantly in the centrilobular region (zone 3) of the liver around the terminal hepatic venule. Localisation of the carboxylesterase can be seen to be more defined in the rat liver section. Centrilobular localisation is a well documented phenomena of drug metabolising enzymes such as the cytochrome P450 system, glutathione-S-transferases and the UDP-glucoronyl S-transferases (Hall *et al.*, 1989). Previous work has also shown that there is a more defined division of labour within the rat liver in comparison to the human liver (Hall *et al.*, 1989). This division is clearly seen within the rat liver section (Figure 5.15B) in comparison to the human liver section (Figure 5.15A).

Taking this data together with the results from the ELISA it can be proposed that an immune response to human carboxylesterase may have an important role in the development and/or perpetuation of halothane hepatitis. Of course the carboxylesterase is only one of up to eight microsomal proteins that become the targets for antibodies in patients' sera and how much of a role the carboxylesterase plays in the pathology of this disease will not be known untill the other human proteins that become trifluoroacetylated are isolated.

### 5.3.5. Trifluoracetylation of human microsomal carboxylesterase.

One important question is what are the factors that cause a protein to become trifluoracetylated and hence lead to an immune response? Using the carboxylesterase as a model protein a few suggestions will be described below. An obvious factor would be that the protein would have to be close to the site of halothane metabolism. Halothane is metabolised under oxidative conditions to a trifluoracetyl radical (Harris *et al.*, 1991). Since the oxidative metabolism of halothane has been found to take place in the microsomal fraction by a P450 mediated reaction (Karashims, 1977), then for a protein to be trifluoracetylated it should be associated with the endoplasmic reticulum of

the cell. The carboxylesterase purified here was from the microsomal fraction of human livers and was found to be localised in the centrilobular region of human and rat liver (Figure 5.15A,B), which is the region where P450s are predominantly found (Hall *et al.*, 1989). Another factor, which has been described above (Section 5.2.8; Figure 5.10), is the relative abundance of the protein. It would seem feasible that the more abundant the protein the greater its chances of being trifluoracetylated. This does indeed appear to be the case for the carboxylestease with its content in the endoplasmic reticulum being estimated to make up approximately 2% of total protein (Figure 5.10). The other proteins that have been identified as being trifluoracetylated are all found at high levels in the endoplasmic reticulum (Kenna *et al* ., 1993). These proteins are GRP94 (100Kda) (Thomassen *et al.*, 1990), ERp72 (76kDa.) (Pumford *et al.*, submitted), calreticulin (63 kDa.) (Butler *et al.*, 1992) and protein disulphide isomerase (57 kDa.) (Kenna *et al.*, 1990). An important observation is that these proteins along with the carboxylesterase are all found within the lumen of the endoplasmic reticulum. (Kenna *et al.* 1992). These proteins all have at their C-terminus the KDEL (Lys-Asp-Glu-Leu), or a related form of this, endoplasmic reticulum retention signal (Pelham, 1990). The sequence derived from the human carboxylesterase cDNA reveals that at its C-terminus there is the related KDEL motif, HIEL (His-Ile-Glu-Leu) (Pelham, 1990). In the 24 amino acids preceding this there are six lysine residues. The work of Harris *et al.* (1991) showed that trifluoracetylation of hepatic proteins occured at lysine residues. It can be postulated that perturbation of this retention signal, by trifluoroacetylation of the lysine residue or of lysine residues near by (in the case of the carboxylesterase), may prevent these proteins being retained within the lumen of the endoplasmic reticulum. This may ultimately lead to the exportation of the protein(s) from the endoplasmic reticulum and ultimately presentation to the immune system. One study has shown that liver biopsies from halothane exposed patients, who did not go on to develope hepatitis, contained trifluoroacetylated proteins (Kenna *et al.*, 1988). This work suggested that all pateints who have undergone halothane anaesthesia produce trifluoroacetylated proteins. A major question still to be answered is how immune sensitisation occurs. Why this should occur in some individuals and not in others that are exposed to halothane is unknown. Other factors that may be of importance include; interindividual variability in the amounts of antigens generated, levels of expression on the hepatocyte surface membrane, antigen-processing and

presentation by antigen-presenting cells, immune recognition and/or specific tolerance to TFA-protien antigens (Kenna *et al.*, 1993). A summary diagram of the above ideas are presented in Figure 5.16.

## 5.4 Summary.

The work in this chapter has described the purification and characterisation of a human hepatic microsomal carboxylesterase. This work has also shown that the human carboxylesterase is an autoantigen in 85% of patients tested with the disease halothane hepatitis (Smith *et al.*, 1993). The purified carboxylesterase and the rabbit antisera produced will be important reagents for the study of this protein both as a xenobiotic metabolising enzyme and as an autoantigen in halothane hepatitis.

The initial characteristion of the enzyme suggested it to be similar to the rat carboxylesterase form pI 6.0 (Mentlein *et al.*, 1980; Harano *et al.*, 1988), and to have similarities to the human 'mid pI' form described by Ketterman *et al.*, (1989). More recently, Kreutz *et al.*, (1993) described the cloning and baculovirus mediated expression of two almost identical carboxylesterases from a liver cDNA library. The difference between these two carboxylesterses is that one, termed hCEv, has two 3bp losses compared to the other, termed hCE. This results in the loss of two amino acids. From the limited $NH_2$-terminal sequence, the specific activities towards p-nitrophenyl acetate and malathione, the electrophoretic mobility and relative abundance it can be presumed that the carboxylesterase purified here is one of those, or a homogenous mixture of those described by Kroetz, *et al.* (1993). Using specific oligonucleotides as probes Kroetz *et al.* (1993) showed that the mRNA for both these proteins was present in human liver samples. From their study the relative expression of the human isozymes appears to vary form individual to individual. Separation of the two proteins by SDS-PAGE and subsequent Western blot analysis will be very difficult since the two proteins differ in size by only 200 Da. Why should humans have two proteins that are practically identical? Maybe, these carboxylesterases are the product of a gene-duplication event.

Much of the future work on carboxylesterases will probably focus on the endogenous role of these enzymes. In rat adipose tissue a microsomal carboxylesterase has already been shown to be fatty acid ethyl ester synthase

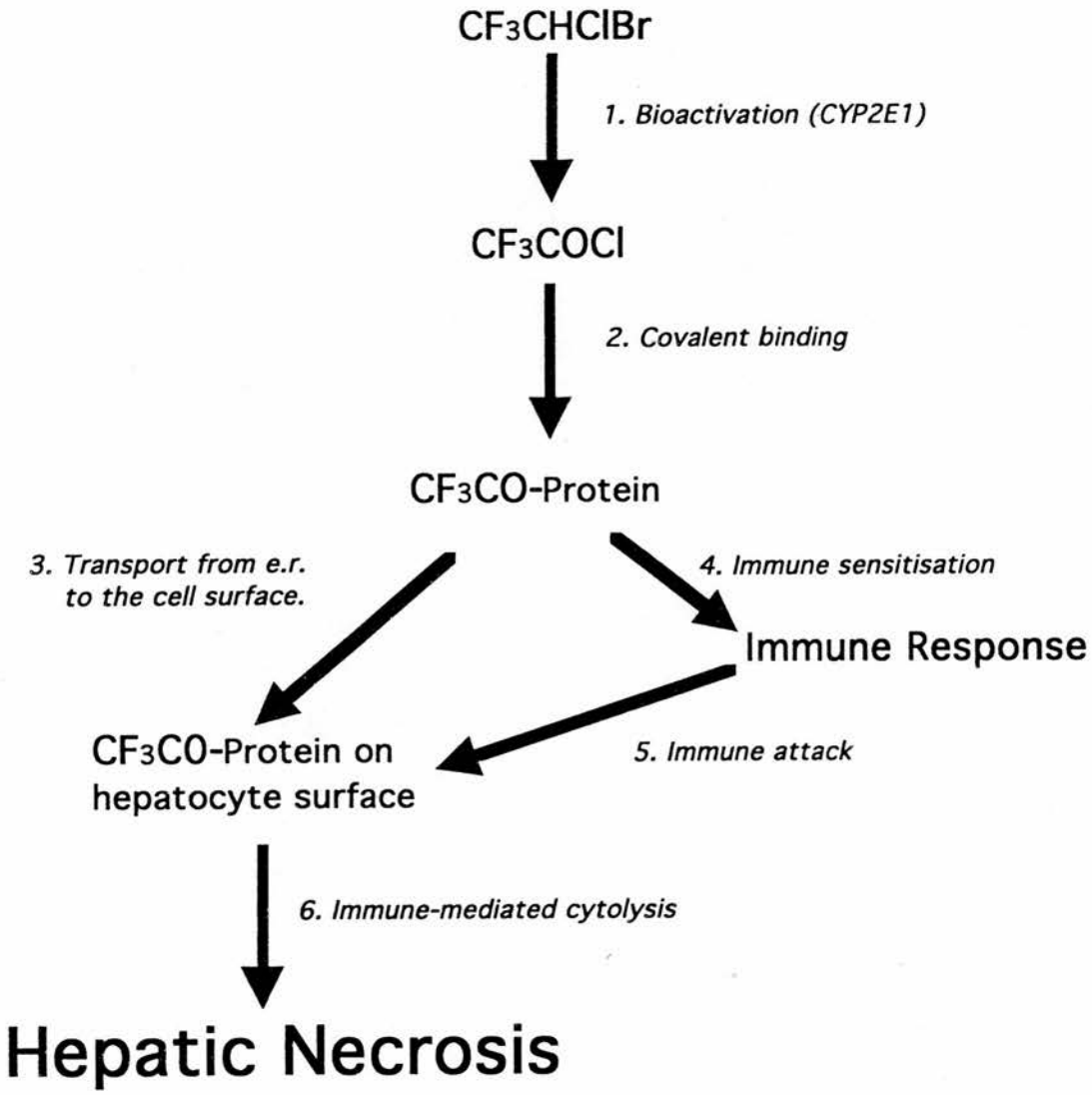



**Figure 5.16. Suggested mechanism of immune-mediated hepatotoxicity.** (From Kenna *et al.*, 1993.)

(Tsujita and Okuda, 1992). A serine protease excreted from human alveolar macrophages (Munger *et al.*, 1991) has identical sequence to the carboxylesterases cloned from a human liver cDNA library (Kroetz *et al.*, 1993).

The carboxylesterases may have some other important physiological role(s), other than xenobiotic metabolism, that has still to be ascribed to them.

The role of the carboxylesterase as an autoantigen in halothane hepatitis has been clearly established from the data presented within this chapter. At present, the diagnosis of halothane hepatitis is largely a clinical one by exclusion of other possible causes of liver damage (Zimmerman, 1978; Walton *et al.*, 1978; Ray and Drummond, 1991). The diagnosis can be confirmed by testing for antibodies in patients' sera that react with trifluoracetylated liver proteins (Kenna *et al.*, 1984 and Kenna *et al.*, 1988) but the assays described are time consuming and technically demanding. The ELISA described here offers promise as a quick, simple and sensitive diagnostic aid. In principle, the assay might also identify patients sensitised to halothane and at risk of hepatitis if re-exposed to this anaesthetic.

The carboxylesterase is only one of up to eight microsomal proteins that are trifluoroacetylated by the oxidative metabolite of halothane. What the relative importance of the carboxylesterase is as an autoantigen in comparison to the other proteins will become clear when the other native proteins become available. This work has served to highlight that autoantibodies are present in the sera of patients with halothane hepatitis that are directed towards the carboxylesterase and not just antibodies to TFA-carboxylesterase as previous work has suggested (Satoh *et al.*, 1989). This discrepency is probably due to the fact that in the work discussd here a human protein was used compared to one derived from rat liver microsomes (Satoh *et al.*, 1989).

In halothane hepatitis the first immune response is most probably against TFA-carboxylesterase. The TFA-carboxylesterase may then come into contact with the immune system, after transport to the cell surface, through perturbation of its C-terminal endoplasmic reticulum retention signal. After this initial immune sensitisation a response to the whole protein may occur. The other proteins that are trifluoracetylated may also go through this series of events. Trifluoracetylation of microsomal proteins has been shown to occur on lysine residues (Harris, 1991). The lysine residues that become trifluoracetylated may be mapped on the carboxylesterase by

trifluoracetylating the protein *in vitro* followed by sequence analysis of peptide fragments obtained after proteolytic digestion of the protein.

The picture that is emerging from studies identifying trifluoracetylated microsomal proteins is that the proteins are abundant, have a long half life and are present within the lumen of the endoplasmic reticulum (Kenna *et al.*, 1993). However, are there other as yet unidentified, proteins trifluoracetylated? For example , some data suggests that a P450 is trifluoracetylated with the appearance of a TFA-P450 occuring quickly and disappearing after 3 hours. This most probably represents the P450 that is involved in the metabolism of halothane, namely CYP2E1 (Kenna, unpublished). Other abundant endoplasmic reticulum proteins, e.g. epoxide hydrolase, maybe modified by the trifluoroacetyl chloride metabolite of halothane. Previous work has centered around Western blotting for identification of antigens, but, as shown here for the human microsomal carboxylesterase, conformational epitopes maybe lost through this technique and only seen via ELISA. Hence, other important autoantigens may yet still be identified.

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**Appendix; Publications arising from this thesis.**

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# Dissection of NADPH–cytochrome P450 oxidoreductase into distinct functional domains

**(drug metabolism/electron transport/flavoproteins/nitric oxide synthase)**

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**ABSTRACT** **NADPH–cytochrome P450 oxidoreductase transfers electrons from NADPH to cytochrome P450 and catalyzes the one-electron reduction of many drugs and foreign compounds. This enzyme is a flavoprotein containing the cofactors FMN and FAD, which are essential for its function. We have expressed the putative FMN and FAD/NADPH binding domains of P450 reductase and show that these distinct peptides fold correctly to bind their respective cofactors. The FAD/NADPH domain catalyzed the one-electron reduction of a variety of substrates but did not efficiently reduce cytochrome *c* or cytochrome P450 (as judged by the oxidation of the CYP1A1 substrate 7-ethoxyresorufin). However, the domains could be combined to provide a functional enzyme active in the reduction of cytochrome *c* and in transferring electrons to cytochrome P450. Both the reconstitution of the domains and the direct binding of cytochrome *c* to the FMN domain were ionic-strength dependent. The FMN domain containing the hydrophobic membrane anchor sequence was a potent inhibitor of reconstituted monooxygenase activity. These data strongly support the hypothesis that FMN/FAD-containing proteins have evolved as a fusion of two ancestral genes and provide fundamental insights into how this and structurally related proteins, such as nitric oxide synthase and sulfite reductase, have evolved and function.**

The microsomal flavoprotein NADPH–cytochrome P450 oxidoreductase (EC 1.6.2.4) shuttles electrons from NADPH via its FMN and FAD prosthetic groups to cytochrome P450 (1). The reductase also has the ability to reduce a host of exogenous electron acceptors, including cytochrome *c*, potassium ferricyanide, and 2,6-dichloroindophenol (DCIP) (2), as well as therapeutically important compounds such as mitomycin c (3) and the benzotriazine SR4233 (4). In addition, P450 reductase is also an electron donor to heme oxygenase (5), the fatty acid elongation system (6), and cytochrome $b_5$ (7) and initiates lipid peroxidation by the one-electron reduction of molecular oxygen (8). P450 reductase was the first protein found to contain FMN and FAD in an equimolar ratio of 1:1 (9). Since its isolation, two other groups of proteins have been identified which contain these flavins as prosthetic groups: (*i*) the nitric oxide synthases, involved in the production of the neurotransmitter, vasodilator, and cytotoxic agent nitric oxide from L-arginine (10, 11), and (*ii*) the $\alpha$ subunit of the bacterial sulfite reductases, which participate in the six-electron reduction of sulfite to sulfide (12). In addition to this similarity, these proteins have been shown to share significant sequence identity, particularly within the suggested functional domains (12, 13).

Initial sequence comparisons of P450 reductase with other proteins reveal a striking homology with two distinct flavoproteins (14, 15). The N-terminal region of the reductase shows homology with the FMN-containing bacterial flavodoxins. The C-terminal portion of the reductase is homologous with the FAD-containing ferredoxin $NADP^+$ reductases. These observations led to the proposal that P450 reductase has evolved as a fusion of two ancestral proteins (15).

We therefore set out to separate the proposed domains of P450 reductase into discrete functional units by expressing specific peptides in *Escherichia coli*. The peptides were designed on the basis that exons or groups of exons within the P450 reductase gene defined specific functional domains, as has been observed for the steroid hormone receptors (16) and the glyceraldehyde phosphate dehydrogenases (17). The ability to dissect P450 reductase into functionally viable domains would provide new avenues for studying the structure and function of this and related proteins.

## MATERIALS AND METHODS

**Constructs.** The cDNA for human NADPH–cytochrome P450 oxidoreductase was obtained from a human skin fibroblast cDNA library (kindly provided by S. M. Keyse, Imperial Cancer Research Fund, Dundee, UK) by PCR. Oligonucleotides for the 5′ (incorporating an *Eco*RI site) and 3′ ends of the reductase cDNA were derived from the sequence reported previously (18). After treatment with Klenow DNA polymerase and digestion with *Eco*RI, the cDNA was ligated into the *Eco*RI/*Sma* I site of pTZ19R (St. Albans, Hertfordshire, UK) and the sequence was confirmed. The human cDNA was the template for further PCR amplifications. Oligonucleotides were generated for the 5′ and 3′ ends of the proposed exons shown in Fig. 1 in order to obtain cDNAs encoding domains of P450 reductase. The exon/intron boundaries were derived from the rat P450 reductase gene (19). The 5′ oligonucleotides were synthesized with an overhanging *Nde* I restriction site whereas the 3′ oligonucleotides contained an *Xho* I site. After amplification and digestion, the cDNAs were ligated into the unique *Nde* I/*Xho* I sites of the expression plasmid pET15b (Novagen). The authenticity of regions generated by PCR was confirmed by sequencing. The plasmids were used to transform *E. coli* BL21(pLysS).

**Purification of the Domains.** Cloning into the *Nde* I/*Xho* I sites of pET15b engineers a $His_6$ linker and a thrombin cleavage site onto the N terminus of the expressed protein. Purification of the His-tagged domains on nickel-agarose columns was carried out according to Novagen's recommendations. BL21 strains harboring the domain expression plasmids were grown at 37°C overnight in LB broth containing ampicillin (50 $\mu$g/ml) and chloramphenicol (34 $\mu$g/ml). Five hundred milliliters of fresh LB broth was inoculated with 5 ml of the overnight culture and the bacteria were grown at 37°C

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Abbreviations: DCIP, 2,6-dichloroindophenol; EROD, 7-ethoxyresorufin O-deethylation.

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to an OD of 0.5–0.6. Isopropyl β-D-thiogalactopyranoside was then added (0.5 mM) and the culture was allowed to grow for 2.5 hr before harvest at 5000 × *g* for 10 min. The pellet from a 500-ml culture was suspended in 20 ml of binding buffer (5 mM imidazole/500 mM NaCl/20 mM Tris·HCl, pH 7.9) and frozen at −70°C for 1 hr. Upon thawing the bacteria were lysed, since the expression strain BL21 contains a plasmid bearing T7 lysozyme. Sonication (MSE probe at full power, 2 × 35 sec with 1 min of incubation on ice between steps) was carried out to shear genomic DNA present in the lysate. The extract was then centrifuged (39,000 × *g* for 30 min) to remove cellular debris and insoluble protein. Bacteria were analyzed for domain expression by SDS/PAGE of soluble and insoluble fractions (data not shown). The FMN/anchor, FMN (no anchor), and FAD/NADPH domains (Fig. 1) produced soluble protein extracts. However, the FAD and FAD/NADPH (small) domains were insoluble, as was the FMN/FAD domain, with only a very small percentage present in the soluble fraction. The supernatant containing the soluble domains was applied onto 2-ml nickel-agarose columns previously equilibrated with binding buffer. Columns were washed with 10 volumes of binding buffer and then 10 volumes of wash buffer (binding buffer containing 20 mM imidazole). The FMN (no anchor) and the FAD/NADPH domains could be eluted from the nickel-agarose column by increasing the imidazole concentration to 60 mM, while the FMN/anchor region was eluted by increasing the imidazole to 300 mM. Insoluble proteins were purified in a manner similar to the soluble domains but in the presence of 6 M urea. Attempts to renature the domains were unsuccessful. The purity of the domains was determined by SDS/PAGE. The insoluble domains were not used in any further experiments. Purified soluble domains were extensively dialyzed against 10 mM potassium phosphate buffer (pH 7.7).

Protein was determined by the method of Lowry *et al.* (20). Thrombin cleavage was performed in 20 mM Tris·HCl, pH 8.4/150 mM NaCl/2.5 mM $CaCl_2$ with a domain/thrombin weight ratio of 5000:1. Cleavage took place at 4°C and was monitored by SDS/PAGE. One hundred percent cleavage occurred after 10 min for the FMN (no anchor) domain and after 4 hr for the FAD/NADPH domain. The $His_6$ tag could not be removed from the FMN/anchor domain. The cut domains were separated from the $His_6$ tag by passing the sample through a nickel-agarose column.

**Spectral Analysis.** Absorption spectra were obtained with a Shimadzu UV 2000 spectrophotometer. FMN and FAD content was calculated after releasing the flavin from the domains by boiling for 3 min in the dark, followed by centrifugation at 20,000 × *g* for 10 min to remove denatured protein. The flavin concentration was determined at 450 nm by using extinction coefficients of 12.2 $mM^{-1}\cdot cm^{-1}$ for FMN (21) and 11.3 $mM^{-1}\cdot cm^{-1}$ for FAD (22).

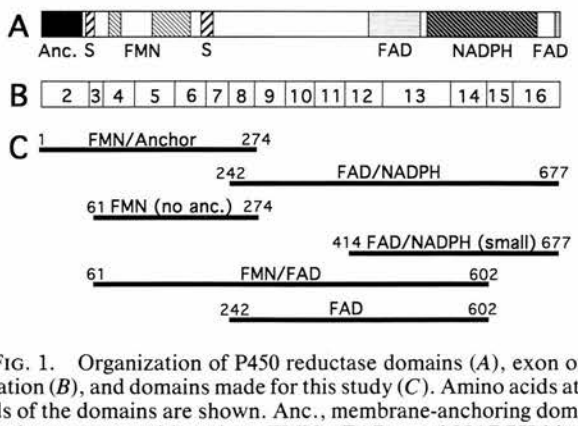


FIG. 1. Organization of P450 reductase domains (*A*), exon organization (*B*), and domains made for this study (*C*). Amino acids at the ends of the domains are shown. Anc., membrane-anchoring domain; S, substrate recognition sites. FMN-, FAD-, and NADPH-binding domains are shown. Calculated molecular masses of the domains: FMN/anchor, 31.1 kDa; FAD/NADPH, 49.7 kDa; FMN (no anchor), 24.2 kDa; FAD/NADPH (small), 30.2 kDa; FMN/FAD, 61.3 kDa; FAD, 41.0 kDa.

**Enzyme Assays.** The one-electron reduction of cytochrome *c*, potassium ferricyanide, DCIP, menadione, and 3-acetylpyridine adenine dinucleotide phosphate was carried out in 50 mM potassium phosphate (pH 7.7) at 37°C (23). For reconstitution of reductase activity, the FMN (no anchor) and the FAD/NADPH domains (0.1 nmol of each) were mixed in 10 mM potassium phosphate (pH 7.7) for 2 hr at 4°C before assay. The FMN/anchor domain was used for the reconstitution of P450 reductase activity. 7-Ethoxyresorufin O-deethylation (EROD) was determined (24) with a Perkin–Elmer LS-3 fluorescence spectrophotometer. Details of the reconstitution assays are in the figure legends. Rat CYP1A1 and P450 reductase were purified as described (25, 26).

## RESULTS

**Expression and Purification of P450 Reductase Domains.** *E. coli* cells were transfected with expression plasmids encoding the P450 reductase domains (Fig. 1). All the peptides tested were expressed at high level (10–15% of total cell protein) but not all were soluble (data not shown). The FAD and the FAD/NADPH (small) domains were totally insoluble and could be purified only under denaturing conditions. The FMN/FAD domain was partially soluble but upon isolation was very susceptible to proteolysis. The FMN (no anchor) domain was predominantly soluble (up to 35 mg of purified protein could be obtained from a 1-liter culture), with the FMN/anchor and the FAD/NADPH domain less so (up to 5 mg of purified protein from a 1-liter culture).

The recombinant His-tagged proteins were purified to homogeneity by nickel-agarose chromatography (Fig. 2). The $His_6$ tag was completely removed from the N terminus of the FMN (no anchor) and FAD/NADPH domains. However, the tag could not be removed from the N terminus of the FMN/anchor domain (data not shown).

**Absorption Spectra of the Oxidized FMN and FAD/NADPH Domains.** The FMN/anchor, FMN (no anchor), and FAD/NADPH domains purified from the bacterial supernatant (under nondenaturing conditions) were yellow, indicating the presence of flavin. To confirm this, UV/visible spectra were recorded. The spectrum of the FMN (no anchor) domain had peaks at 370 and 453 nm and a broad absorption band between 570 and 630 nm (probably due to some reduced FMN being present in this sample) (Fig. 3*A*). This spectrum was virtually identical to that reported by Kurzban *et al.* (27) for the FAD-depleted P450 reductase and similar to that predicted from the computer model of Oprian and Coon (28). The spectrum of the FAD/NADPH domain had absorption maxima at 382 and 454 nm (Fig. 3*B*), similar to the FMN-depleted preparations of P450 reductase (29, 30). However, this spectrum had more definition than the FMN-depleted reductase produced by site-directed mutagenesis (31). Analysis of flavin content showed that the FMN/anchor domain contained 0.67 mol of FMN per mol of protein, and the FMN domain, 0.63:1. The FAD/NADPH domain contained 0.70 mol of FAD per mol of protein. When the FMN and FAD/NADPH domains were combined in a 1:1 ratio, a spectrum identical to that of native P450 reductase was obtained (Fig. 3 *C* and *D*).

The above data showed that the FMN and FAD domains could be expressed separately and could fold independently to bind their respective cofactors. To investigate whether the FAD/NADPH protein could transfer electrons to the FMN (no anchor) domain to produce an air-stable semiquinone similar to the native P450 reductase (Fig. 3*C*), the two domains were mixed and an excess of NADPH was added. A spectrum characteristic of the air-stable semiquinone form o

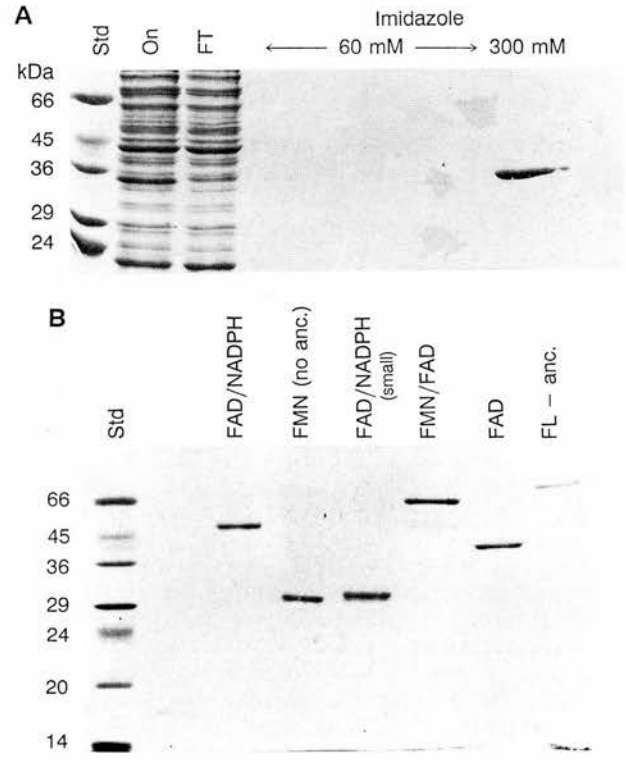


FIG. 2. Expression of P450 reductase domains in *E. coli*. The cDNA sequences encoding the domains shown in Fig. 1 were expressed in *E. coli* BL21(pLysS) using the pET15b vector. Domains were purified to apparent homogeneity on nickel-agarose columns. (*A*) Purification of the FMN/anchor domain. Std, molecular size standards; On, soluble fraction applied to the column (15 $\mu$g); FT, flowthrough (15 $\mu$g); 300 mM, domain eluted with 300 mM imidazole after washing of the column with 60 mM imidazole (2 $\mu$g). (*B*) SDS/12% PAGE analysis of the other purified domains (1 $\mu$g) illustrated in Fig 1. FL − anc., full-length P450 reductase minus the anchor domain.

native P450 reductase was obtained (Fig. 3 *C* and *D*). This slowly reoxidized over a period of 4 hr, which is unlike intact P450 reductase, where this intermediate is stable for up to 48 hr. This reduction occurs via electron transfer from NADPH through FAD (30). The similarity of the spectra (Fig. 3 *C* vs. *D*), which are characteristic of FMN semiquinone, indicates that electrons are transferred from FAD to the FMN domain. This is supported by the finding that the FAD semiquinone rapidly reoxidized (25 min in a similar experiment using the FAD/NADPH domain; data not shown) and that NADPH did not directly reduce the FMN (no anchor) domain (data not shown).

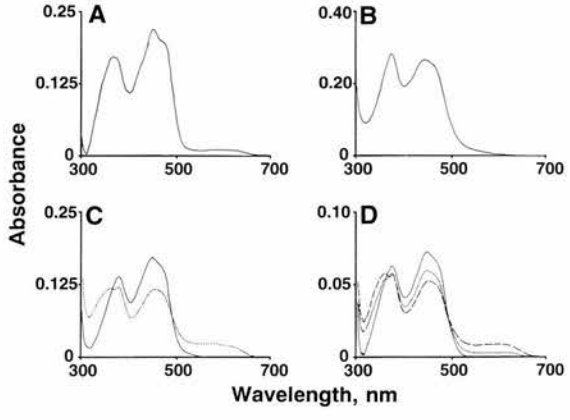


FIG. 3. Absorption spectra of purified P450 reductase domains. (*A*) FMN (no anchor) domain, 55 $\mu$M. (*B*) FAD/NADPH domain, 50 $\mu$M. (*C*) Native P450 reductase, 10 $\mu$M: ——, oxidized; - - - -, air-stable semiquinone 24 hr after addition of 200 $\mu$M NADPH. (*D*) Spectrum obtained on mixing the FMN (no anchor) (7.5 $\mu$M) and FAD/NADPH (7.5 $\mu$M) domains: ——, oxidized; — —, reduced spectrum 30 min after the addition of 200 $\mu$M NADPH; - - - -, reduced spectrum 4 hr after the addition of 200 $\mu$M NADPH.

**Interaction Between the FMN and FAD/NADPH Domains.** In view of the above observation, we determined whether the two domains could bind directly to each other. The His-tagged FMN (no anchor) domain was bound to a nickel-agarose column and the FAD/NADPH domain (without the $His_6$ tag) applied. The FAD/NADPH domain became tightly bound to the column, but only when the FMN domain was bound. The FAD/NADPH domain could be dissociated from the FMN (no anchor) domain and eluted by increasing the ionic strength of the column buffer (Fig. 4), indicating that ionic interactions were important for binding to occur.

In addition, the ability of the domains to bind cytochrome *c* was studied. When cytochrome *c* was applied to the column containing the immobilized FMN (no anchor) domain it was retained on the column. This could also be eluted by increasing the ionic strength of the buffer (data not shown). In a similar experiment with the FAD/NADPH protein immobilized on a nickel-agarose column, cytochrome *c* did not bind (data not shown).

**Functional Analysis of Domains and Reconstitution of Activity.** The above data indicated that the FAD/NADPH and FMN domains folded correctly and could interact to form a complex with each other. We then established whether the domains could couple to form a catalytically active unit. The catalytic activity of the FAD/NADPH domain alone was also assessed.

The FAD/NADPH domain could reduce a range of one-electron acceptors and showed transhydrogenase activity in the absence of the FMN domain but could not efficiently reduce cytochrome *c* (Table 1). The activities measured were to a varying degree decreased relative to native P450 reductase. Activity toward these one-electron acceptors was not significantly increased by the presence of the FMN (no anchor) domain. However, cytochrome *c* reductase activity was reconstituted when a combination of the FMN (no anchor) and FAD/NADPH domains was used. The activity of the reconstituted domains was found to be around 2% that of the native enzyme.

To test for the reconstitution of cytochrome P450 monooxygenase activity, the rat cytochrome P450 CYP1A1 was incorporated into incubation mixtures containing the FMN/anchor and FAD/NADPH domains. Activity was monitored as the rate of EROD. In this case a functional monooxygenase system could be reconstituted. Virtually no activity was measured when the FMN/anchor domain was omitted, but when it was replaced by the FMN (no anchor) domain some

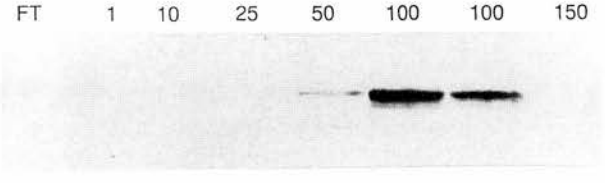


FIG. 4. Interaction of the FMN (no anchor) domain with the FAD/NADPH domain. His-tagged FMN (no anchor) domain (2 mg) was bound to a 1-ml nickel-agarose column and the FAD/NADPH domain (0.1 mg/ml) was applied in 1 mM potassium phosphate (pH 7.7). This resulted in the binding of the latter domain. The concentration of the buffer passed through the column was then increased from 1 to 150 mM in a stepwise fashion and the resulting eluates were analyzed by Western blotting using an antibody to full-length P450 reductase. FT, flowthrough.

Table 1. Specific activities of P450 reductase and domains

| Domain(s) | Specific activity (%) | | | | | |
|---|---|---|---|---|---|---|
| | $Fe(CN)_6^{3-}$ | DCIP | Menadione | 3-AcPyADP* | CYP1A1 | Cytochrome *c* |
| P450 reductase | 33.1 | 11.2 | 7.67 | 1.14 | 6.34 | 23.5 |
| FAD/NADPH | 17.9 (54.1) | 0.456 (4.1) | 0.145 (1.9) | 0.228 (20) | 0.001 (0.016) | 0.025 (0.11) |
| FAD/NADPH plus FMN | 16.1 (48.6) | 0.705 (6.3) | 0.134 (1.7) | 0.207 (18) | 0.121 (1.9) | 0.410 (1.7) |
| FMN | – | – | – | – | – | – |

Specific activities are expressed as μmol of substrate reduced per min per mg of protein, except for menadione, where the oxidation of NADPH was followed, and CYP1A1 activity, which was determined as EROD activity and is expressed as nmol of resorufin produced per min per nmol of P450. All assays were carried out in triplicate with SEM < 5%.
*3-Acetylpyridine adenine dinucleotide phosphate.

deethylation was measured (around 20% of that seen with the FMN/anchor; data not shown). Also, when P450 was not present no activity was measured. The reconstitution was time dependent (Fig. 5*A*). Reconstitution of cytochrome *c* reductase activity was also time dependent (data not shown). The ability to reconstitute P450 reductase activity was ionic-strength dependent (Fig. 5*B*).

To establish whether the FMN/anchor domain could interact with P450 to affect the coupling of P450 reductase to P450, the effect of adding this domain to a reconstituted system containing native P450 reductase and CYP1A1 was investigated. Preincubation of CYP1A1 with the FMN/anchor domain resulted in almost complete inhibition of EROD activity (Fig. 6). Fifty percent inhibition was observed at a domain/native reductase ratio of ≈4:1. The FAD/NADPH domain had little effect on EROD activity, although a reproducible slight decrease in activity was seen at the lowest domain concentration tested. An interesting result was observed when titrating the FMN (no anchor) domain into the reconstituted system. This domain was found to activate EROD activity up to 3-fold. This appeared to be specific, as the denatured FMN domain or free FMN did not cause this effect (data not shown).

## DISCUSSION

We describe the dissection of an FMN/FAD-containing flavoprotein, NADPH–cytochrome P450 oxidoreductase, into structurally and functionally independent domains. This provides strong experimental evidence to substantiate the hypothesis that this protein has evolved from two distinct ancestral genes (15). Indeed, many of the properties of the ancestral proteins appear to have been retained and there is no need for direct interaction between the two domains for FMN and FAD binding. However, for stabilization of the incorporated flavin in the protein (i.e., to maintain a 1:1 molar ratio FAD or FMN to protein) and for efficient electron transfer, interactions may be needed between the domains and/or flavins.

The expressed FAD/NADPH domain could catalyze the reduction of certain compounds, indicating correct folding of both the FAD and NADPH binding regions. Certain reaction rates were comparable to those of the native enzyme, indicating that the FAD/NADPH domain is at least in part responsible for the one-electron reduction of many compounds, including redox cycling drugs. The much slower rate of reduction of substrates such as menadione by the FAD/NADPH domain indicates that for certain compounds this region is not the major site of reduction. Previous work on the FMN-depleted native P450 reductase also indicated that this was the case (29, 30). The FAD/NADPH and FMN domains could be reconstituted to form a complex active in the reduction of cytochrome *c* and in donating electrons for cytochrome P450-dependent monooxygenase reactions. This fascinating finding demonstrated that electron transfer between the domains, although not optimal, could be achieved. Studies into why these interactions are so sensitive to ionic strength, and studies into optimal domain size should identify some of the structural and functional requirements needed for efficient transfer between the peptides.

Comparison of the FMN-binding region of P450 reductase with the flavodoxin from *Desulfovibrio vulgaris* shows that highest homology lies within exons 4–6 (19). However, we chose to express exons 2–8 and exons 3–8 as the FMN-binding domain. In recent experiments we have expressed only exons 4–6 but found that this protein did not bind FMN. Similarly, sequence alignments based on the crystal structure

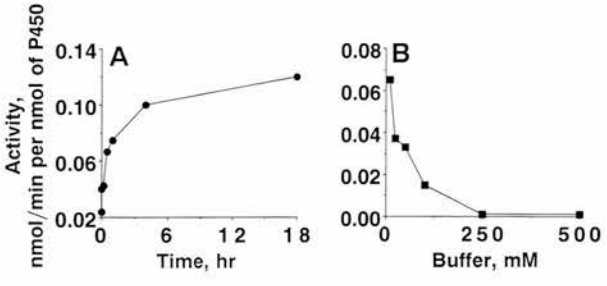


FIG. 5. (*A*) Reconstitution of cytochrome P450 monooxygenase activity. The FMN/anchor and FAD/NADPH domains (0.1 nmol) were mixed in 10 mM potassium phosphate (pH 7.7) in a volume of 20 μl at 4°C. After various times the domains were incubated with dilauroyl phosphatidylcholine (25 μg) and CYP1A1 (37.5 pmol in 5 μl) at 37°C for 5 min before assay EROD activity (24). (*B*) Effect of ionic strength on the reconstitution of EROD. Domains were preincubated, as described for *A*, for 2 hr in various concentrations of potassium phosphate buffer at pH 7.7. Dilauroyl phosphatidylcholine (25 μg) and CYP1A1 (37.5 pmol in 5 μl) were then added and incubated at 37°C for 5 min before assay for EROD.

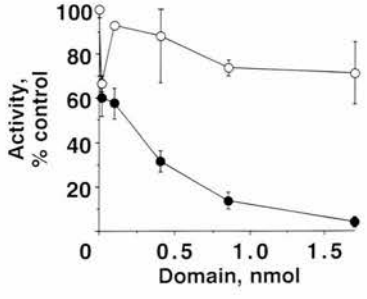


FIG. 6. Inhibition of monooxygenase activity by the FMN/anchor domain of P450 reductase. CYP1A1 (7.5 pmol) and 25 μg of dilauroyl phosphatidylcholine were incubated for 5 min with various concentrations of the FMN/anchor (●) or FAD/NADPH (○) domain (0.04–1.7 nmol) in 47.5 μl at 37°C for 5 min. Native P450 reductase (0.05 nmol in 2.5 μl) was then added and the sample was incubated for 5 min before measurement of EROD activity. Results (mean ± SEM, $n = 3$) are expressed as percentage of the activity in the absence of the domain; 100% = 6.34 nmol/min per nmol of P450.

of spinach ferredoxin $NADP^+$ reductase have revealed that the minimal FAD/NADPH-binding region of the protein should be contained within exons 12–16 (32). However, expression of exons 12–16 yielded an insoluble protein which, from its white appearance, did not bind FAD. In other cases, even when insoluble products were formed, the color of the protein suggested that flavin had been incorporated.

Expression of the FAD-binding region of P450 reductase, excluding the proposed NADPH-binding domain (exons 15 and 16) (Fig. 1), generated an insoluble protein that did not bind FAD. Alignment of this region with spinach ferredoxin $NADP^+$ reductase shows that exons 15 and 16, in addition to containing important amino acids for $NADP^+$ binding, contain residues involved in covering the face of the flavin without hydrogen bonding to it (32). It has also been suggested that the large contact surface between the FAD- and NADPH-binding domains would not allow them to be functionally separated (32). These observations would explain why the FAD domain did not fold in the absence of the NADPH domain.

The binding between the FMN (no anchor) and FAD/NADPH domains, the interaction of the FMN (no anchor) domain with cytochrome *c*, and the reconstitution of monooxygenase activity were all ionic-strength dependent. These findings are probably related to the highly charged nature of P450 reductase, which contains 97 Asp and Glu residues and 74 Arg and Lys residues out of a total of 677 amino acids. The direct involvement of the FMN (no anchor) domain with cytochrome *c* binding and in its reduction is in agreement with previous studies (29, 31, 33). Crosslinking studies of native P450 reductase with cytochrome *c* indicate that amino acids 207–215 of the reductase are involved in the binding between the two proteins (33). These residues are located in the FMN domain. Various amino acids within the FMN domain have also been implicated in P450 interactions (34).

The FMN/anchor domain was found to be a potent inhibitor of reconstituted monooxygenase activity. This could be explained by the domain preventing the association of the P450 to P450 reductase. This suggests a role for the hydrophobic anchor in directing P450 reductase to the P450, as suggested in earlier studies (23, 35). However, the $His_6$ tag and thrombin cleavage site could not be removed from this domain. It cannot be ruled out that the $His_6$ tag may affect the association of the domain with the bilayer and that the inhibitory effect we observe may be occurring outside the bilayer. The FMN (no anchor) domain did not inhibit but significantly increased monooxygenase activity. This could be explained if the FMN (no anchor) domain can accept electrons from the FAD of native P450 reductase and supply electrons directly to P450.

Our data support the hypothesis that P450 reductase has evolved as a fusion of two ancestral proteins (15). Indeed, we may speculate that as two independent proteins the ancestors of P450 reductase functioned as a dehydrogenase/electron-transferase in a primitive organism. By fusion of the genes encoding these ancient flavoproteins a more catalytically efficient electron-transfer system was produced.

The ability to dissociate the protein into domains will be of significant value for x-ray and NMR studies as well as in understanding how the domains interact with each other and with cytochrome P450 to form a functional electron-transfer unit. Such studies will provide insights into the function of structurally related proteins such as nitric oxide synthase and sulfite reductase.


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# Autoantibodies to hepatic microsomal carboxylesterase in halothane hepatitis

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Halothane hepatitis can be life-threatening, and this severe adverse reaction may arise via an immune process. We have detected autoantibodies to purified human liver microsomal carboxylesterase in sera of 17 out of 20 patients with halothane hepatitis (85%) but not in 9 halothane-exposed controls and in only 2 (at low levels) of 33 patients with liver disease due to other causes. Immunohistochemical studies localised the carboxylesterase predominantly to the centrilobular region of liver sections, which is consistent with the area affected by halothane hepatitis. Human hepatic microsomal carboxylesterase is a target antigen in halothane hepatitis, and an immune response to this protein may be involved in the liver damage observed.

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Halothane causes two types of hepatotoxicity. The milder form is characterised by minor increases in serum transaminases, develops in 20% or so of patients, and has been attributed to cytotoxic effects of halothane metabolites.[1,2] The severe form (halothane hepatitis) is much rarer (1 in 10 000) but it can lead to massive hepatic necrosis and death.[1,2] Halothane hepatitis may arise via an immune response to halothane-modified (trifluoroacetylated) liver proteins[2] and a similar mechanism could underlie the very rare hepatotoxicity caused by enflurane and isoflurane.[3] Several of the implicated trifluoroacetylated proteins have been purified from halothane-treated rats and characterised biochemically.[2] One of them, a purified 59 kDa rat protein, corresponds to a carboxylesterase isozyme.[4] We have purified the equivalent carboxylesterase isozyme from pooled human livers (unpublished) and report here that carboxylesterase autoantibodies are present in the sera of patients with halothane hepatitis.

Sera from 20 patients with halothane hepatitis, defined clinically as otherwise unexplained hepatitis within 28 days of halothane anaesthesia, were studied. They were aged 32–60 (median 53) and 17 were female. All had had halothane anaesthesia before, on between 1 and 8 occasions (median 2), and the interval between the current and previous or latest prior exposure ranged from 7 days to 10 years (median 6 weeks). 13 patients had liver failure and 12 died. Control sera were obtained from 9 patients who had received halothane several times without liver damage; from 18 healthy blood donors; and from 11 patients with fulminant hepatic failure (6 paracetamol, 2 hepatitis B, 1 hepatitis A, 2 non-A, and non-B hepatitis), 6 with autoimmune chronic active hepatitis, 4 with alcoholic liver disease, 8 with primary biliary cirrhosis, and 4 with jaundice due to flucloxacillin.

Purification and characterisation of human liver carboxylesterase and production of a monospecific polyclonal antiserum will be described elsewhere. Antibodies to carboxylesterase were determined by enzyme-linked immunosorbent assay.[5] Sera were screened at a dilution of 1 in 100, using a polyclonal sheep anti-human IgG coupled to horseradish peroxidase (Scottish Antibody Production Unit, Lanark), diluted 1 in 1000, as secondary antibody. Sera were regarded as antibody positive when optical density (OD) values at 492 nm were more than 2 SD above the mean for the blood donors (OD > 0·211). Immunohistochemical analysis was done on formalin-fixed, paraffin-embedded liver tissue.[6]

Antibodies to human liver carboxylesterase were detected in 17 patients with halothane hepatitis (85%) but not in 9 halothane-exposed patients with no liver disease (figure 1). Low levels were detected in 2 patients with primary biliary cirrhosis but all the 31 other patients with non-halothane liver disease were negative. Immunohistochemical studies with rabbit polyclonal antiserum revealed that the human liver carboxylesterase was present throughout the liver but was concentrated in the centrilobular region (figure 2). The pattern of damage commonly observed in patients with halothane hepatitis is also centrilobular.[1] These findings suggest that an immune response to human carboxylesterase may have an important role in the development and/or perpetuation of halothane hepatitis.

Microsomal carboxylesterase is one of a group of at least eight hepatic microsomal proteins that interact covalently with trifluoroacetylchloride, the major reactive metabolite produced in the livers of laboratory animals and patients exposed to halothane at normal oxygen tensions.[2,4,8] Furthermore, immunohistochemical studies with rabbit antiserum specific for the covalently bound trifluoroacetyl hapten show that, in livers of halothane-treated rats, the trifluoroacetylated proteins are concentrated in the centrilobular region.[7] Presumably it is trifluoroacetylated carboxylesterase that provides the immunogenic stimulus that, in susceptible individuals, gives rise to an antibody response.

We do not know why such an antibody response is seen in patients with halothane hepatitis but not in halothane-exposed patients in whom hepatitis does not develop. All exposed patients metabolise halothane to trifluoroacetylchloride and produce trifluoroacetylated protein antigens.[9] Susceptibility factors may include individual variability in the level of expression of the cytochrome P450 isozyme responsible for bioactivation of halothane (probably CYP2E1[2]), in the level of expression of the protein targets, in the presentation of antigens to the immune system, and/or in the immune response itself.

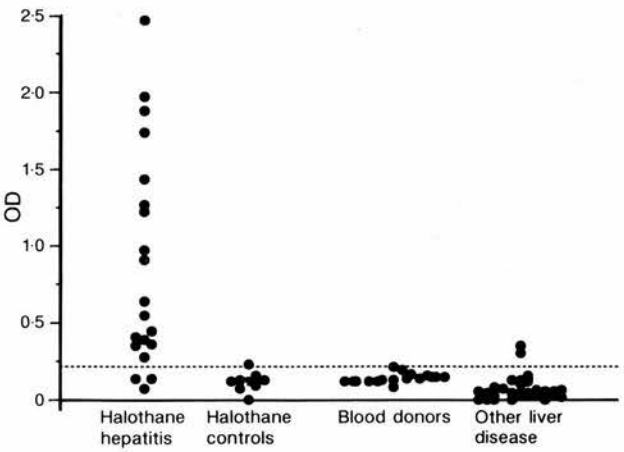


Figure 1: **Detection of antibodies to carboxylesterase in patients' sera by ELISA**

Dotted line shows upper limit of normal control range.

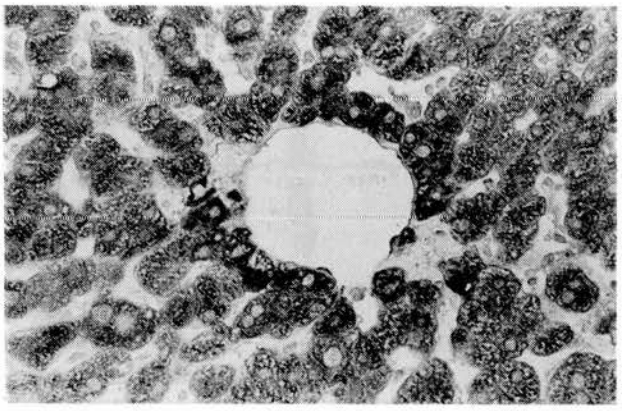

Figure 2: **Immunohistochemical staining of human liver section with rabbit polyclonal antiserum to human liver carboxylesterase**

The carboxylesterase we used was purified from liver obtained from a pool of individuals with no recent exposure to halothane so the reactivity detected by ELISA must have been due to recognition of native, non-trifluoroacetylated epitopes—ie, the patients' antibodies are true autoantibodies. Experiments with immunoblotting[8] have indicated that the major epitopes on rat hepatic trifluoroacetylated proteins which are recognised by antibodies from patients with halothane hepatitis are the trifluoroacetyl-hapten together with essential structural features unique to the protein carriers.[4,8] In general the patients' antibodies did not recognise non-trifluoroacetylated rat proteins.[4,8] Sequence identity for human and rat hepatic carboxylesterases is only 77% (unpublished) so the non-trifluoroacetylated epitopes recognised by the patient's antibodies may not be found in the rat protein—or the epitopes may be conformational, detectable by ELISA but destroyed by the harsh conditions of immunoblotting.

The diagnosis of halothane hepatitis is largely a clinical one, by exclusion of other possible causes of liver damage. The diagnosis can be confirmed by testing for antibodies to trifluoroacetylated liver proteins[2,5,8] but the assays described are technically demanding. A carboxylesterase ELISA offers promise as a quick, simple, and sensitive test. In principle, the assay might also identify patients previously sensitised to halothane and at risk of hepatitis if re-exposed.

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